II. REMARKS

Formal Matters

Claims 1-21 and 23 are pending after entry of the amendments set forth herein.

Claims 1-23 were examined and were rejected. Claims 24-60 were withdrawn from consideration.

Claims 1-15 and 23 are amended. The amendments to claims 1-11, 13, and 14 were made solely in the interest of expediting prosecution, and are not to be construed as an acquiescence to any objection or rejection of any claim. The amendments to claims 2-9 and 11-15 and 23 are editorial in nature; accordingly, no new matter is added by these amendments. Support for the amendments to claims 1 and 10 is found in the claims as originally filed, and throughout the specification, in particular at the following exemplary locations: claim 1: "heterologous to the host microorganism": page 7, lines 10-12; "transformed host microorganism": page 8, lines 8-13; "results in production of IPP": page 20, lines 26-29; page 2, lines 22-24; Example 3 (page 28, lines 10-15); Figure 2; Example 4; " wherein the host microorganism is a prokaryote that does not normally synthesize IPP through the mevalonate pathway": page 4, line 29 to page 5, line 1; and page 15, lines 9-20; and claim 10: page 10, line 11 to page 14, line 7. Accordingly, no new matter is added by these amendments.

Claims 22 and 24-60 are canceled without prejudice to renewal, without intent to acquiesce to any rejection, and without intent to surrender any subject matter encompassed by the canceled claims. Applicants expressly reserve the right to pursue any canceled subject matter in one or more continuation and/or divisional applications.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Rejections withdrawn

Applicants note with gratitude that the following rejections, raised in the Office Action mailed May 5, 2004, have been withdrawn: 1) rejection of claims 13-21 and claims 1-23 under 35 U.S.C.§112, second paragraph; and 2) rejection of claims 3 and 4 under 35 U.S.C.§103(a).

Examiner Interviews

The undersigned Applicants' representative thanks Examiner Fronda and Examiner Achutamurthy for the courtesy of two telephonic interviews which took place on October 26, 2004 and on December 7, 2004. The October 26, 2004 Examiner Interview was attended by Examiners Fronda and Achutamurthy, inventor Dr. Jack Newman, and Applicants' representative Paula A. Borden. The December 7, 2004 Examiner Interview was attended by Examiner Fronda, inventor Dr. Jack Newman, and Applicants' representative Paula A. Borden. The undersigned Applicants' representative thanks the Examiners for their time and consideration of this application, as well as helpful suggestions regarding claim language.

During the interviews, the rejection of claims 1-4, 6-8, 10, and 12-23 under 35 U.S.C.§ 112, first paragraph; and the rejection of claims 1, 12, 13, 22, and 23 under 35 U.S.C.§102(b), were discussed.

Rejection under 35 U.S.C.§112, first paragraph

Claims 1-4, 6-8, 10, and 12-23 were rejected under 35 U.S.C.§112, first paragraph, as allegedly failing to comply with the written description requirement.

The Office Action stated that the specification does not provide a written description of any other genus members other than SEQ ID NO:1, which encodes an acetoacetyl CoA thiolase; SEQ ID NO:2, which encodes an HMG-CoA synthase; SEQ ID NO:3, which encodes an HMG-CoA reductase; SEQ ID NO:4, which encodes a mevalonate kinase; SEQ ID NO:5, which encodes a phosphomevalonate kinase; SEQ ID NO:6, which encodes a mevalonate pyrophosphate decarboxylase; and SEQ ID NO:10, which encodes isopentenyl pyrophosphate isomerase. Applicants respectfully traverse the rejection.

As discussed in more detail below: i) a number of nucleotide sequences encoding various mevalonate pathway enzymes were known as of the priority date of the instant application; ii) the fact that various mevalonate pathway enzymes from diverse organisms were functionally interchangeable in mevalonate-producing organisms and/or retained enzymatic function in non-mevalonate-producing organisms had become well accepted by those skilled in the art as of the priority date of the instant application. Accordingly, those skilled in the art, given the instant specification, would have recognized that Applicants were in possession of the invention as claimed, as of the priority date of the instant application.

Comments regarding satisfying the written description requirement of 35 U.S.C.§112, first paragraph

To satisfy the written description requirement of 35 U.S.C.§112, first paragraph, an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date, he or she was in possession of the claimed invention.

The Guidelines for Examination of Patent Applications Under the 35 U.S.C. §112, paragraph 1 "Written Description" Requirement (Federal Register 66, No. 4, January 5, 2001; "Written Description Guidelines") provides instructions for examining patent applications for compliance with the written description requirement of 35 U.S.C.§112, first paragraph.

The Written Description Guidelines state that the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species; and that a "representative number of species" means that the species which are adequately described are representative of the entire genus. The Written Description Guidelines state that there may be situations in which one species adequately supports a genus; and that what constitutes a "representative number" is an inverse function of the skill and knowledge in the art.[1] The Patent Office has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims. MPEP §2163.

As stated in the MPEP §2163, information which is well known in the art need not be described in detail in the specification.[2] Providing citations to literature sources which disclose mevalonate pathway enzyme-encoding nucleotide sequences, where the nucleotide sequences were known in the art as of the priority date of the instant application, suffices for compliance with the written description requirement of 35 U.S.C.§112, first paragraph. In the following section, Applicants direct the Examiner's attention to those passages in the specification and to a large number of scientific literature references that amply support Applicants' position that the claims find written description support in the specification.

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[1] Written Description Guidelines, page 1106.

[2] *In re Buchner* 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.* 231 USPQ 81, 94 (Fed. Cir. 1986); and *Lindemann Maschinenfabrik GmbH v. American Hoist & Derrick Co.* 221 USPQ 481, 489 (Fed. Cir. 1984).

The specification describes an adequate number of species.

The specification provides a description of nucleotide sequences encoding acetoacetyl CoA thiolase. SEQ ID NO:1 is a nucleotide sequence encoding acetoacetyl CoA thiolase from *Escherichia coli*. The specification also cites references that provide nucleotide sequences encoding acetoacetyl CoA thiolase from *Ralstonia eutrophus*, and *Saccharomyces cerevisiae*. Specification, page 10, line 24 to page 11, line 2. Providing citations to literature sources which disclose acetoacetyl CoA thiolase-encoding nucleotide sequences suffices for compliance with the written description requirement of 35 U.S.C.§112, first paragraph.

Furthermore, additional nucleotide sequences encoding acetoacetyl CoA thiolase from various organisms were known as of the December 6, 2001 filing date of the instant application. For example, GenBank Accession No. AF113522 (July 20, 1999) provides the nucleotide sequence encoding an acetoacetyl CoA thiolase from *Zea mays*; GenBank Accession No. D00512 (February 1, 2000) and Fukao et al. ((1989) *J. Biochem.* 106 (2):197-204) provide the nucleotide sequence encoding an acetoacetyl CoA thiolase from rat; and GenBank Accession No. S70154 (August 23, 1994) and Song et al. ((1994) *Biophys. Res. Commun.* 201 (1), 478-485) provide the nucleotide sequence encoding an acetoacetyl CoA thiolase from human.

The specification provides a description of nucleotide sequences encoding hydroxymethylglutaryl-CoA synthase (HMGS). SEQ ID NO:2 is a nucleotide sequence encoding an HMGS from *S. cerevisiae*. The specification cites a reference that provides nucleotide sequences encoding HMGS from *Blattella germanica*. Specification, page 11, lines 12-17. Providing citations to literature sources which disclose HMGS-encoding nucleotide sequences suffices for compliance with the written description requirement of 35 U.S.C.§112, first paragraph.

Furthermore, additional nucleotide sequences encoding HMGS from various organisms were known as of the December 6, 2001 filing date of the instant application. For example, GenBank Accession No. AB037666 and Teak et al. ((2000) *J. Bacterial.* 182 (15), 4153-4157) provide nucleotide sequences encoding an HMGS from *Streptomycin* sp. strain CL190; GenBank Accession No. X83618 and Mascara et al. ((1995) *Arch. Biochem. Biophys.* 317 (2), 385-390) provide nucleotide sequences encoding an HMGS from *Homo sapiens*; GenBank Accession No. U90884 and Ate et al. ((1990) *Proc. Natl. Acad. Sic. U.S.A.* 87 (10), 3874-3878) provide nucleotide sequences encoding an HMGS from *Sues scrota*; GenBank Accession No. AF406763 (August 16, 2001) provides a nucleotide sequence encoding an HMGS from *Streptococcus thermopiles*; GenBank Accession No. M33648 (April 27, 1993) and Ate et al. ((1990) *Proc. Natl. Acad. Sic. U.S.A.* 87 (10):3874-3878) provide nucleotide sequences encoding

an HMGS from *Rates norwegicus*; GenBank Accession No. M11869 (April 27, 1993) and Gil et al. ((1986) *J. Biol. Chem.* 261:3710-3716) provide nucleotide sequences encoding an HMGS from *Mesocricetus auratus*; GenBank Accession No. M60657 (March 6, 1995) and Kattar-Cooley et al. ((1990) *Arch. Biochem. Biophys.* 283 (2), 523-529) provide nucleotide sequences encoding an HMGS from *Gallus gallus*.

The specification provides a description of nucleotide sequences encoding hydroxymethylglutaryl-CoA reductase (HMGR). SEQ ID NO:3 is a nucleotide sequence encoding a truncated version of HMGR from *S. cerevisiae*. The specification cites references that provide nucleotide sequences encoding HMGR from *Sulfolobus solfataricus*, *Haloferax volcanii*, and *S. cerevisiae*. Specification, page 11, line 26 to page 12, line 8. Providing citations to literature sources which disclose HMGR-encoding nucleotide sequences suffices for compliance with the written description requirement of 35 U.S.C.§112, first paragraph.

Furthermore, additional nucleotide sequences encoding HMGR from various organisms were known as of the December 6, 2001 filing date of the instant application. For example, GenBank Accession No. AB037666 and Tagaki et al. ((2000) *J. Bacteriol.* 182 (15), 4153-4157) provide nucleotide sequences encoding an HMGR from *Streptomyces* sp. strain CL190; GenBank Accession No. L19349 (July 26, 1993) provides nucleotide sequences encoding an HMGR from *Dictyostelium discoideum*; GenBank Accession No. L19261 (April 20, 1994) provides nucleotide sequences encoding an HMGR from *Arabidopsis thaliana*; GenBank Accession No. L76979 and Lum et al. ((1996) *Yeast* 12 (11), 1107-1124) provide nucleotide sequences encoding an HMGR from *Schizosaccharomyces pombe*; GenBank Accession No. L78791 and Pe et al. ((1997) *J. Biol. Chem.* 324, 619-626) provide nucleotide sequences encoding an HMGR from *Trypanosoma cruzi*; GenBank Accession No. X58370 (July 11, 2000) and Corrochano and Avalos ((1992) *Exp. Mycol.* 16:167-171) provide nucleotide sequences encoding an HMGR from *Gibberella fujikuroi*; and GenBank Accession No. U43711 (June 6, 2000) and Jain et al. (2000) *Plant Mol. Biol.* 42:559-569) provide nucleotide sequences encoding an HMGR from *Morus alba*.

The specification provides a description of nucleotide sequences encoding mevalonate kinase (MK). SEQ ID NO:4 is a nucleotide sequence encoding a MK from *S. cerevisiae*. Nucleotide sequences encoding MK from various organisms were known as of the December 6, 2001 filing date of the instant application. For example, GenBank Accession No. AB037666 and Tagaki et al. ((2000) *J. Bacteriol.* 182 (15), 4153-4157) provide nucleotide sequences encoding an MK from *Streptomyces* sp. strain CL190; GenBank Accession No. AF141853 and Lluch et al. ((2002) *Plant Mol. Biol.* 42 (2), 365-

376) provide nucleotide sequences encoding an MK from *Arabidopsis thaliana*; GenBank Accession No. AF137598 and Houten et al. ((1999) *Hum. Mol. Genet.* 8 (8), 1523-1528) provide nucleotide sequences encoding an MK from mouse; GenBank Accession No. M88468 and Schafer et al. ((1992) *J. Biol. Chem.* 267 (19), 13229-13238) provide nucleotide sequences encoding an MK from *Homo sapiens*; GenBank Accession No. AF429384 (Oct. 25, 2001) provides a nucleotide sequence encoding an MK from *Hevea brasiliensis*; GenBank Accession No. AJ279018 (August 16, 2001) provides a nucleotide sequence encoding an MK from *Lactobacillus helveticus*; and GenBank Accession No. AF290099 (August 29, 2000) and Wilding et al. ((2000) *J. Bacteriol.* 182:4319-4327) provide nucleotide sequences encoding an MK from *Streptococcus pneumoniae*.

The specification provides a description of nucleotide sequences encoding phosphomevalonate kinase (PMK). SEQ ID NO:5 is a nucleotide sequence encoding a PMK from *S. cerevisiae*. Nucleotide sequences encoding PMK from various organisms were known as of the December 6, 2001 filing date of the instant application. For example, GenBank Accession No. L77214 and Chambliss et al. ((1996) *J. Biol. Chem.* 271 (29), 17330-17334) provide nucleotide sequences encoding PMK from *Sus scrofa*; GenBank Accession No. AB037666 and Tagaki et al. ((2000) *J. Bacteriol.* 182 (15), 4153-4157) provide nucleotide sequences encoding a PMK from *Streptomyces* sp. strain CL190; Gen Bank Accession No. L77213 and Chambliss et al. ((1996) *J. Biol. Chem.* 271 (29), 17330-17334) provide nucleotide sequences encoding a PMK from *Homo sapiens*; GenBank Accession No. AJ279018 (August 16, 2001) provides nucleotide sequences encoding a PMK from *Lactobacillus helveticus*; GenBank Accession No. AF290099 and Wilding et al. ((2000) *J. Bacteriol.* 182 (15), 4319-4327) provide nucleotide sequences encoding a PMK from *Streptococcus pneumoniae*; GenBank Accession No. AJ279018 (August 16, 2001) provides a nucleotide sequence encoding a PMK from *Lactobacillus helveticus*; and GenBank Accession No. AF290097.1 (August 29, 2000) and Wilding et al. ((2000) *J. Bacteriol.* 182 (15), 4319-4327 provide nucleotide sequences encoding a PMK from *Streptococcus pyogenes*.

The specification provides a description of nucleotide sequences encoding mevalonate pyrophosphate decarboxylase (MPD). SEQ ID NO:6 is a nucleotide sequence encoding an MPD from *S. cerevisiae*. Nucleotide sequences encoding MPD from various organisms were known as of the December 6, 2001 filing date of the instant application. For example, GenBank Accession No. U49260 and Toth and Huwler ((1996) *J. Biol. Chem.* 271 (14), 7895-7898) provide nucleotide sequences encoding an MPD from *Homo sapiens*; GenBank Accession No. U53706 (May 18, 1996) provides nucleotide sequences encoding an MPD from rat; GenBank Accession No. AB037666 and Tagaki et al. ((2000) *J. Bacteriol.* 182 (15), 4153-4157) provide nucleotide sequences encoding an MPD from

Streptomyces sp. strain CL190; GenBank Accession No. AJ279018 (August 16, 2001) provides a nucleotide sequence encoding an MPD from *Lactobacillus helveticus*; GenBank Accession No. AF290099 (August 29, 2000) provides a nucleotide sequence encoding an MPD from *Streptococcus pneumoniae*; GenBank Accession No. AF290097 (August 29, 2000) provides a nucleotide sequence encoding an MPD from *Streptococcus pyogenes*; and GenBank Accession No. AF290095 (August 29, 2000) provides a nucleotide sequence encoding an MPD from *Enterococcus faecium*.

Nucleotide sequences encoding mevalonate pathway enzymes from various organisms were known in the art as of the filing date of the instant application; and were known to be functionally interchangeable in mevalonate-producing organisms and/or to retain enzymatic function in non-mevalonate-producing organisms.

As noted above, nucleotide sequences encoding mevalonate pathway enzymes from various organisms were known in the art as of the filing date of the instant application; and were known to be functionally interchangeable in mevalonate-producing organisms and/or to retain enzymatic function in non-mevalonate-producing organisms. Furthermore, a number of nucleotide sequences encoding various mevalonate pathway enzymes were known as of the December 6, 2001 filing date of the instant application to be functionally interchangeable in mevalonate-producing organisms and/or to retain enzymatic function in non-mevalonate-producing organisms.

As explained by Dr. Newman during the October 26, 2004 telephonic interview, as of the December 6, 2001 priority date, a number of nucleotide sequences encoding various enzymes in the mevalonate pathway were known by those skilled in the art to be functionally interchangeable in mevalonate-producing organisms and/or to retain enzymatic function in non-mevalonate-producing organisms. The following is a discussion of journal articles published before December 6, 2001, which showed that mevalonate pathway enzymes from a first organism are fully functional in a second organism, where the second organism is of a different genus and species from the first organism. A copy of each of the journal articles is provided herewith.

acetoacetyl CoA thiolase

1) Vollack and Bach ((1996) *Plant Physiol.* 111(4):1097-107; "Vollack"; a copy of which is provided herewith as Exhibit 1)

Figure 1 of Vollack shows that an acetoacetyl CoA thiolase (also referred to as "ERG10" or "AtoB") from radish is functional in a *Saccharomyces cerevisiae* (yeast) mevalonate pathway. The data

show that the acetoacetyl CoA thiolase from radish is functionally equivalent to the acetoacetyl CoA thiolase from *S. cerevisiae*.

2) Peoples and Sinskey ((1989) *J Biol Chem*.264(26):15298-303; "Peoples"; a copy of which is provided herewith as Exhibit 2)

Peoples shows that an acetoacetyl CoA thiolase (encoded by a gene designated as "*PhbA*") from *Alcaligenes eutrophus* is functional in *E. coli*, like the *atoB* gene used in the reconstituted mevalonate pathway in the instant application. Peoples is cited in the instant application as an example of an acetoacetyl CoA thiolase-encoding nucleic acid that can be used in a mevalonate pathway.

HMGS

3) Montamat et al. ((1995) *Gene* 167:197-201; "Montamat"; a copy of which is provided herewith as Exhibit 3)

Montamat shows that an HMGS from *Arabidopsis thaliana* (thale cress/mouse-ear cress) is functional in an *S. cerevisiae* mevalonate pathway. Figure 3 of Montamat shows that a plant-derived HMGS can function in place of a yeast HMGS.

HMGR

4) Kato-Emori et al. ((2001) *Mol Genet Genomics* 265(1):135-42; "Kato-Emori"; a copy of which is provided herewith as Exhibit 4)

Kato-Emori shows that a plant HMGR is functionally equivalent to a yeast HMGR. Specifically, Figure 3 of Kato-Emori shows that HMGR from the plant *Cucumis melo* (melon) functions in place of *S. cerevisiae* HMGR in the yeast mevalonate pathway.

5) Learned and Fink ((1989) *Proc Natl Acad Sci U S A* 86(8):2779-83; "Learned"; a copy of which is provided herewith as Exhibit 5)

Learned shows that an HMGR from *Arabidopsis thaliana* is functional in an *S. cerevisiae* mevalonate pathway. Specifically, Figure 4 of Learned shows complementation of a non-functional yeast HMGR gene with a functional *A. thaliana* HMGR gene.

mevalonate kinase

6) Riou et al. ((1994) *Gene* 148(2):293-7; "Riou"; a copy of which is provided herewith as Exhibit 6)

Riou shows that an MK from *Arabidopsis thaliana* is functional in place of a yeast MK in an *S. cerevisiae* mevalonate pathway. Specifically, Table 1, and Figures 1 and 2 of Riou show that an MK from *A. thaliana* can functionally replace yeast MK.

mevalonate phosphate decarboxylase

7) Cordier et al. ((1999) *Plant Mol Biol.* 39(5):953-67; "Cordier"; a copy of which is provided herewith as Exhibit 7)

Cordier shows that a MPD from *Arabidopsis thaliana* is functional in an *S. cerevisiae* mevalonate pathway. Specifically, Figure 4 of Cordier shows complementation of the non-functional yeast MPD gene with an MPD gene from *A. thaliana*. The amino acid sequence of *Arabidopsis thaliana* MPD exhibits only about 55% identity with the yeast, human and rat MPDs; yet the *Arabidopsis thaliana* MPD can functionally substitute for *S. cerevisiae* MPD.

The fact that the various mevalonate pathway enzymes from various organisms were functionally interchangeable in mevalonate-producing organisms and/or retained enzymatic function in non-mevalonate-producing organisms had become well accepted by those skilled in the art as of the priority date of the instant application.

Conclusion as to the rejection under 35 U.S.C.§112, first paragraph

As discussed in ample detail above, several nucleotide sequences encoding mevalonate pathway enzymes from various organisms were known as of the December 6, 2001 filing date of the instant application. A number of such sequences are provided in the instant specification and/or literature references providing such sequences were provided in the instant specification. Information that is well known in the art need not be described in detail in the specification. Furthermore, it was well known as of the December 6, 2001 filing date of the instant application, that mevalonate pathway enzymes from diverse organisms were functionally interchangeable in mevalonate-producing organisms and/or retained enzymatic function in non-mevalonate-producing organisms. Accordingly, those skilled in the

art, given the instant disclosure, in combination with the knowledge in the art, would have recognized that Applicants were in possession of the invention as claimed as of the December 6, 2001 filing date. As such, the instant specification is in compliance with the written description requirement of 35 U.S.C.§112, first paragraph.

Applicants submit that the rejection of claims 1-4, 6-8, 10, and 12-23 under 35 U.S.C. §112, first paragraph, has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

Rejection under 35 U.S.C.§102(b)

Claims 1, 12, 13, 22, and 23 were rejected under 35 U.S.C. §102(b) as allegedly anticipated by Hoshino et al. (EP 0955363; "Hoshino").

The Office Action stated that Hoshino teaches using transformed host cells for the production of products including isopentenyl pyrophosphate containing vectors or plasmids comprising genes encoding enzymes in the mevalonate pathway. Applicants respectfully traverse the rejection.

The instant invention as claimed differs from Hoshino in a number of ways. First, the instant claims recite a method for producing IPP via the mevalonate pathway, the method involving culturing a transformed host microorganism in a suitable medium, where the transformed host microorganism is one that comprises one or more **heterologous** nucleic acids encoding two or more mevalonate pathway enzymes. Secondly, the instant claims recite a method for producing IPP via the mevalonate pathway, the method involving culturing a transformed host microorganism in a suitable medium, where the transformed host microorganism is one that comprises one or more heterologous nucleic acids encoding a **plurality** (i.e., two or more) of mevalonate pathway enzymes into a host microorganism, such that IPP is produced in the transformed host microorganism. These differences are discussed in more detail below.

As discussed during the December 7, 2004 telephonic interview with Examiner Fronda, Hoshino discusses the following:

- introduction of a nucleic acid comprising *Phaffia rhodozyma* (a yeast) mevalonate pathway enzyme-encoding nucleotide sequence into *P. rhodozyma*. This amounts to introduction of **homologous** nucleotide sequences, **not heterologous** nucleotide sequences, as required by claim 1; and
- introduction of a nucleic acid comprising a nucleotide sequence encoding a single *P. rhodozyma* mevalonate pathway enzyme into *E. coli*. This is introduction of a heterologous nucleotide sequence; however, it is **not** introduction of a nucleic acid comprising nucleotide sequences encoding a **plurality (two or more)** mevalonate pathway enzymes, as required by claim 1. Furthermore, as discussed in more detail below, introduction of a nucleic acid comprising a nucleotide sequence encoding a single *P. rhodozyma* mevalonate pathway enzyme individually into *E. coli*, or even introduction of a nucleic acid comprising nucleotide sequences encoding all of the enzymes listed in paragraph 0005 of Hoshino (HMGS, HMGR, MK, and MPD) into *E. coli*, **will not result** in production of IPP, as required by claim 1. This is because *E. coli* **lacks an endogenous mevalonate pathway**, and therefore cannot provide the missing enzymes.

These points are discussed in more detail below.

1) Hoshino neither discloses nor suggests producing IPP via the mevalonate pathway in a transformed host microorganism that comprises one or more heterologous nucleic acids encoding two or more enzymes in the pathway.

Hoshino discusses introduction of a nucleic acid comprising a *Phaffia rhodozyma* (a yeast) mevalonate pathway enzyme-encoding nucleotide sequence into *P. rhodozyma*. This is discussed in paragraphs 0005 and 0029-0035 of Hoshino. As stated in Hoshino, the purpose of carrying out such transformations is to enhance the enzymatic activity of a rate-limiting reaction in the mevalonate pathway. See, e.g., paragraph 0029 of Hoshino.

Introduction of a *P. rhodozyma* mevalonate enzyme-encoding nucleotide sequence into *P. rhodozyma* is **not** introduction of a **heterologous** nucleic acid into a host microorganism, as required by claim 1. Instead, introduction of *P. rhodozyma* mevalonate enzyme-encoding nucleotide sequences into *P. rhodozyma* is introduction of **homologous** nucleotide sequences into *P. rhodozyma.*

In each case, the introduction suggested by Hoshino is performed in *P. rhodozyma.* See, for example, Hoshino at paragraph 0005 ("These genes may be amplified in a suitable host, such as *P. rhodozyma*); paragraph 0032 ("Transformed *P. rhodozyma*"); paragraph 0033 ("...and then introduced into *P. rhodozyma*"); paragraph 0034 ("And such a vector can be introduced into *P. rhodozyma* ...). Finally, the conclusion to all of these examples of genetic engineering then begins, in paragraph 0035 ("Such a genetically engineered *P. rhodozyma*...").

2) Hoshino neither discloses nor suggests producing IPP via a mevalonate pathway in a transformed host microorganism, where the transformed host microorganism comprises one or more heterologous nucleic acids comprising nucleotide sequences encoding a plurality (i.e., two or more) mevalonate pathway enzymes, such that IPP is produced in the transformed host microorganism.

Hoshino discusses introduction of nucleotide sequences encoding single *P. rhodozyma* mevalonate pathway enzymes individually into *E. coli*, such that a single *P. rhodozyma* mevalonate pathway enzyme is produced in the transformed *E. coli.* This is discussed in paragraphs 0005, 0020-0027; and Examples 7, 9, and 11 of Hoshino. This introduction was solely to produce the mevalonate pathway enzymes individually in the transformed *E. coli*, so that the enzymes could be individually characterized *in vitro.* Hoshino, Examples 7, 9, and 11.

Hoshino does not disclose introducing into a host microorganism one or more heterologous nucleic acids comprising nucleotide sequences encoding two or more mevalonate pathway enzymes, as required by claim 1, such that IPP is in the transformed host microorganism, as required by claim 1.

To understand the differences between Hoshino and the instant invention as claimed, it is essential to understand that *E. coli* (and many other prokaryotes) **does not have an endogenous mevalonate pathway**.

Hoshino states that the invention involves "cloning and determination of the genes which code for HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, mevalonate pyrophosphate decarboxylase and FPP synthase" and "the enzymatic characterization as a result of the expression of such genes in suitable host organisms such as *E. coli*. Hoshino, paragraph 0005. However, as noted above, *E. coli* lacks an endogenous mevalonate pathway. Thus, if one introduced nucleic acids containing nucleotide sequences encoding each of HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, and mevalonate pyrophosphate decarboxylase into *E. coli*, but did not supply the other enzymes in the mevalonate pathway (which were not disclosed in Hoshino), such **would not result in production of IPP,** because *E. coli* **lacks endogenous mevalonate pathway enzymes that would provide the missing enzymes**.

The final Office Action stated that although not all of the enzymes recited in claim 1 are disclosed in Hoshino, the transformed host cells taught by Hoshino inherently have the endogenous acetoacetyl-CoA thiolase, phosphmevalonate kinase, and mevalonate pyrophosphate decarboxylase. However, as noted above, this is not true for cells, such as *E. coli*, that lack endogenous mevalonate pathway.

As discussed above, Hoshino neither discloses nor suggests culturing a transformed host microorganism in a suitable medium, where the transformed host microorganism is one that comprises one or more heterologous nucleic acids comprising nucleotide sequences encoding two or more enzymes in the mevalonate pathway into a host microorganism, such that IPP is produced in the transformed host microorganism. Accordingly, Hoshino cannot anticipate claims 1, 12, 13, 22, and 23.

Nevertheless, and solely in the interest of expediting prosecution, claim 1 is amended to recite "wherein the host microorganism is a prokaryote that does not normally synthesize IPP through the mevalonate pathway." Because *P. rhodozyma* is not a prokaryote, any disclosure in Hoshino relating to *P. rhodozyma* is not relevant to claim 1 as amended. Hoshino neither discloses nor suggests a method of culturing a transformed host microorganism in a suitable medium, where the transformed host microorganism is one that comprises one or more heterologous nucleic acids comprising nucleotide sequences encoding two or more enzymes in the mevalonate pathway into a host microorganism, **and where the host microorganism is a prokaryote that does not normally synthesize IPP through the**

mevalonate pathway, such that IPP is produced in the transformed host microorganism.

Conclusion as to the rejection under 35 U.S.C.§102(b)

Applicants submit that the rejection of claims 1, 12, 13, 22, and 23 under 35 U.S.C. §102(b) has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

III. CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number BERK-036.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: Feb. 9 2005

By: _____
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Plant Physiol. (1996) 111: 1097–1107

Cloning of a cDNA Encoding Cytosolic Acetoacetyl-Coenzyme A Thiolase from Radish by Functional Expression in *Saccharomyces cerevisiae*[1,2]

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A cDNA coding for radish (*Raphanus sativus* L.) acetoacetyl-coenzyme A thiolase (AACT) was cloned by complementation of the *erg10* mutation affecting AACT in yeast (*Saccharomyces cerevisiae*). The longest reading frame encodes a protein of 406 amino acids with a predicted relative molecular weight of 42,032, with significant similarities to eukaryotic and prokaryotic thiolases. There is no evidence for the presence of a leader peptide characteristic, e.g. of glyoxysomal thiolase. Yeast transformants expressing the radish AACT gene placed under the control of the GAL1 promoter exhibited a 10-fold higher enzyme activity than a wild-type yeast strain after induction by galactose. This enzyme activity is exclusively localized in the soluble fraction but not in membranes. These data indicate that we have cloned a gene encoding cytoplasmic (biosynthetic) AACT. Genomic DNA gel blot analysis suggests the presence of a single AACT gene, which is expressed in all parts of the seedling. Expression in cotyledons appears to be light-stimulated. We present preliminary evidence that a smaller transcript represents an antisense species being read from the same gene.

For yeast and mammalian tissue, it has been well documented (see Bach et al., 1990, 1991, and literature cited therein) that the conversion of three units of acetyl-CoA to HMG-CoA is catalyzed by two enzymes: AACT (acetyl-CoA acetyl transferase, EC 2.1.3.9), catalyzing a Claisen-type condensation, and HMGS ([*S*]-3-hydroxy-3-methylglutaryl-CoA:acetoacetyl-CoA lyase, CoA acylating, EC 4.1.3.5), catalyzing an aldol condensation. HMG-CoA serves two functions: first, as the substrate for mevalonate biosynthesis, catalyzed by HMGR (3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate:$NADP^+$ oxidoreductase, CoA acylating, EC 1.1.1.34), and second, as a putative intermediate in the degradation of Leu via HMG-CoA lyase ([*S*]-3-hydroxy-3-methylglutaryl-CoA lyase, EC 4.1.3.4).

Two different types of thiolase are found in both eukaryotes and in prokaryotes: AACT (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (= OACT, EC 2.3.1.16). OACT (also called thiolase I) has a broad chain-length specificity for its substrates and is involved in degradation pathways, such as fatty acid β-oxidation. AACT (also called thiolase II) is specific for the thiolysis of acetoacetyl-CoA and is involved in biosynthetic pathways such as poly-β-hydroxybutyrate synthesis in certain bacteria (see Murphy, 1994; Lee et al., 1995, and refs. cited therein) or isoprenoid biogenesis in eukaryotes. In eukaryotes, there are two forms of OACT, one located in mitochondria and the other located in peroxisomes (for literature citations, see Igual et al., 1992; Kanayama et al., 1994). In yeast, the formation of acetoacetyl-CoA appears to be an important step in the regulation of growth (Kornblatt and Rudney, 1971) and of ergosterol biosynthesis (Trocha and Sprinson, 1976; Servouse and Karst, 1986).

Until recently, little was known about the enzymology and genetics of the reactions leading from acetyl-CoA to mevalonic acid via HMG-CoA in plants (Gray, 1987; Kleinig, 1989; Bach et al., 1990, 1991; Alam et al., 1991; Van der Heijden et al., 1994). Although genes encoding various plant HMGR isozymes have been cloned from several species (for recent literature citations, see Enjuto et al., 1994; Stermer et al., 1994; Chappell, 1995; Weissenborn et al., 1995) according to the databases, full-length cDNA clones of plant genes encoding biosynthetic AACT have not been characterized. While this paper was under revision, an Arabidopsis cDNA sequence with significant similarities to vertebrate HMGS was made available (accession no. X83882, Montamat et al., 1995).

For the cloning of plant AACT, the complementation technique was chosen, using suitable *erg* (ergosterol-

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Abbreviations: AACT, acetoacetyl-CoA thiolase; DIG, digoxygenin; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; OACT, 3-oxoacyl-CoA thiolase; PQQ, pyrroloquinoline quinone; Ura, uracil.

deficient) and thermolabile yeast mutants (Karst and Lacroute, 1974). These *erg* mutants, which are impaired in AACT, consist of two linked complementation units, *erg10A* and *erg10B* (Servouse et al., 1984). The conditional *erg10* mutant has been successfully used to clone an AACT gene from the industrial yeast *Saccharomyces uvarum* (Dequin et al., 1988b). More recently, the AACT gene was isolated from *Saccharomyces cerevisiae* (Hiser et al., 1994) using another yeast *erg10* mutant. This paper describes the isolation of a radish (*Raphanus sativus* L.) cDNA encoding the cytosolic AACT by complementation of the temperature sensitivity of the *erg10A* mutation in yeast.

MATERIALS AND METHODS

Strains and Media

Escherichia coli strains DH10B (BRL) and XL1-Blue MRF' (Stratagene) were used as plasmid or phage hosts. Yeast strains F2sp5 [*MATa, erg10A, ura3–52, his3-D200, leu2–3,112, gal2*$^-$] and W303–1B [*MATα, ura3–52, his3-D200, leu2–3,112, trp, ade*] were kindly provided by F. Karst (Université de Poitiers, France). F2sp5 is derived from strain FL100, which is not able to grow on Gal. Therefore, it was crossed with W303–1B (Thomas and Rothstein, 1989) to produce ergosterol-auxotrophic yeast strains, in which plant cDNAs placed under the control of the *GAL1* promotor can be expressed, depending on the ability to use Gal as a carbon source (Schneider and Guarente, 1991). Sporulation was induced according to the protocol of Sprague (1991). Meiosis and sporulation of diploids and random spore analysis were carried out as described by Kassir and Simchen (1991) and by Dawes and Hardie (1974), respectively. The resulting haploids were replica-plated and incubated (at either 28 or 37°C) on a medium containing Gal to select them for the trait of thermolability that is linked to AACT deficiency and the phenotype of ergosterol auxotrophy. Thus, strain KV5 [MATa, *erg10A, ura3–52, his3-D200, leu2–3,112, Gal2*$^+$] was chosen for the following complementation experiments. Media for the growth of yeast were as described by Sherman (1991): YPGlu contained 2% Glc; YPGal contained 2% Gal; and YNB consisted of 0.67% yeast nitrogen base with ammonium sulfate, 2% Glc, and appropriate supplements for selection. Ergosterol was dissolved in detergent (Tergitol NP-40 [Sigma]:ethanol (1:1, v/v) and was added to liquid and solid media at a concentration of 4 and 80 μg/mL, respectively, for the propagation of ergosterol-auxotrophic yeast strains F2sp5 and KV5.

cDNA Library Construction

Conditions for the hydroponic culture of radish (*Raphanus sativus* L.) seedlings were as described previously (Bach and Lichtenthaler, 1983). Total RNA from 10 g of 4-d-old etiolated radish seedlings (cotyledons and stems without roots) was extracted according to Dean et al. (1985). Radish mRNA was enriched by oligo(dT)-cellulose affinity chromatography (Aviv and Leder, 1972), slightly modified as a batch procedure. Radish cDNA was synthesized from 5 μg of mRNA using the cDNA SYNTHESIS kit from Stratagene with some modifications. After blunt-end conversion, *Xba*I linkers were ligated to both ends of the cDNAs. Double digestion with restriction endonucleases *Xho*I and *Xba*I created an *Xba*I site upstream and an *Xho*I site downstream of the cDNAs, respectively. Digested or excess linkers and adaptors, as well as cDNA fragments smaller in size than 500 bp, were removed by chromatography on a 1-mL Sephacryl S-500HR spin column. A total yield of approximately 600 ng of cDNA was estimated from the color intensity on ethidium bromide-containing agarose plates. *Xho*I/*Xba*I-predigested arms of λMAX1 from Clontech (1 μg) were ligated to 100 ng of radish cDNA and in vitro packaged with phage particles of the Stratagene GiGApack Gold kit. The primary titer of the library was determined as 1.5×10^6 independent clones with an average insert size of 0.9 kb. The library was amplified in *E. coli* XL1-Blue MRF', resulting in a titer of 2.2×10^9 plaque-forming units mL^{-1}. The conversion from the λMAX1 phage library to the pYEUra3 plasmid library was performed as described in the user's manual of the Exassist/Solr System from Stratagene. Plasmid DNA isolation followed standard procedures (Sambrook et al., 1989). Plasmid DNA was purified using fast-performance liquid chromatography on a Superose 6 prep-grade column purchased from Pharmacia (McClung and Gonzales, 1989). Alternatively, plasmid DNA was purified using Qiagen (Chatsworth, CA) columns according to the manufacturer's protocol.

Complementation of the *S. cerevisiae erg10* Mutation

About 10 μg from the radish cDNA library were used to transform AACT-deficient yeast strain KV5 by the lithium acetate method as previously described (Becker and Guarente, 1991), but without carrier DNA in the transformation assays. Ura3$^+$-transformants were pooled, and aliquots were plated on YPGal-complete medium. The Petri dishes were incubated at 37°C to select transformants expressing Gal-induced radish AACT, and thus complementing the mutation, which causes thermolability. Plasmid DNA was recovered (Kaiser and Auer, 1993) from six yeast transformants that showed thermoresistance after induction by Gal and were transformed into *E. coli* DH10B by electroporation. Restriction analysis revealed all six clones carrying an identical 1.5-kb cDNA (referred to as cRS10) inserted into the *Xho*I/*Xba*I cloning site of pYEUra3. The corresponding plasmid, referred to as pYRS10, was then retransformed into the yeast strain KV5, thus generating strain KV10 to confirm the Gal-dependent phenotype of thermoresistance at 37°C in 100% of the transformants.

DNA Sequencing and Sequence Analyses

pcRS10 was constructed by ligating the 1.5-kb *Xho*I/*Xba*I fragment cRS10 into pBluescript (SK$^+$) for sequence analysis by the dideoxynucleotide method (Sanger et al., 1977) using Hyperpaper^{35}S (Amersham). The complete sequence of cRS10 was determined with a set of two flanking pBluescript (SK$^+$) primers (T3 and M13–20) by creating nested deletion mutants of cRS10 with exonuclease III. Double-

stranded plasmid DNA was sequenced using Sequenase 2.0 from United States Biochemical or *Taq* polymerase from Promega's fmol cycle sequencing kit, both according to the manufacturers' instructions. The nucleic acid sequence of cRS10 and the deduced amino acid sequence were used to search database libraries for homologies with the DNASIS/PROSIS software from Hitachi (Tokyo, Japan). Further sequence analyses were performed at the Institut de Biologie Moléculaire des Plantes (Strasbourg, France) with the BLAST network service (Altschul et al., 1990). Sequence alignments and additional analyses were performed using the Genetics Computer Group sequence analysis software package (version 7.0, April 1991) for the Vax workstation installed at the Institut de Biologie Moléculaire des Plantes.

RNA Isolation and RNA Gel Blot Analyses

Total RNA from radish tissue was isolated according to the protocol of Groppe and Morse (1993), developed for RNase-rich marine mollusks, with some modifications. Approximately 10 g of plant material were deep-frozen in liquid nitrogen and pulverized with a mortar and pestle. Additional steps in the protocol were adjusted to this quantity of tissue. DNA prepared in parallel could be precipitated by means of isopropanol and was used for genomic DNA gel blot analyses.

For the isolation of RNA from yeast, cultures of 100 mL (A_{695} = 1.5) were centrifuged in 50-mL Falcon (Becton Dickinson) tubes (4000*g*, 5 min) and washed twice with diethyl pyrocarbonate-treated (RNase-free) water. The pellet was resuspended in 10 mL of lysis buffer (Groppe and Morse, 1993) plus 10 mL of phenol, followed by the addition of 10 mL of glass beads (diameter 0.5 mm). Cells were broken by vortexing (5 × 1 min, maximum speed, interrupted by 1-min cooling periods on ice). After that, 2 mL of chloroform:isoamyl alcohol (49:1, v/v) were added, and the sample was processed further, as described by Groppe and Morse (1993).

cDNA probes were labeled by the incorporation of nonradioactive DIG-11-dUTP using random-primed labeling, according to the manufacturer's protocol (Boehringer Mannheim). Oligonucleotides were end-labeled with DIG-dUTP by means of terminal dideoxynucleotide transferase (Schmitz et al., 1991).

Sense and antisense mRNA species were detected with DIG-UTP-labeled RNA probes that exhibited single-strand target specificity. They were labeled with the hapten DIG by the aid of the DIG RNA labeling kit from Boehringer Mannheim, according to the manufacturer's protocol, and pcRS10 (a derivative of pBluescript SK) as the template. T3 RNA polymerase thus generated an antisense-specific RNA probe, and T7 RNA polymerase generated a sense-specific probe.

Total RNA (usually 10 μg per lane) was electrophoretically separated in 1.2% agarose gels (Sambrook et al., 1989). However, the concentration of formaldehyde was reduced to 0.41 M (see Chomczynski, 1992). Downward alkaline capillary transfer to positively charged nylon membranes (Appligene, Heidelberg, Germany) followed the protocol of Chomczynski (1992). Hybridizations and detection of DIG-labeled probes were carried out as described by Engler-Blum et al. (1993). The usual hybridization and washing temperature was 68°C (60°C when oligonucleotide probes were used). Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Tropix, Bedford, MA) was used as a chemiluminescent substrate for membrane-based detection of alkaline-phosphatase conjugates (Düring, 1991).

Genomic DNA Gel Blot Analysis

For genomic DNA gel blot analyses, 10 μg of each genomic DNA were digested with various restriction endonucleases and electrophoretically separated in 0.8% agarose gels. λDNA was used as a size marker, digested with *Hin*dIII and *Hin*dIII/*Eco*RI. The gel was washed with 0.5 M HCl for 15 min to depurinate the DNA, which facilitates the transfer. Capillary transfer, hybridization, and signal detection were carried out as described for the RNA gel blot analyses.

Determination of AACT Activity in Yeast Transformants

Yeast strain KV10, carrying the radish cDNA cRS10 under control of the *GAL1* promoter, the mutant strain KV5, and strain W303–1B as a reference were used to inoculate liquid cultures with 100 mL of minimal medium supplemented with ergosterol and with either Glc or Gal as a carbon source. The cultures were incubated at 26°C. The cells were harvested by means of centrifugation at 5000*g* for 5 min, at which point they had reached an A_{600} of 1.5. Disruption of yeast cells by glass beads, preparation of protein extracts, and membrane fractionation were performed as previously described (Vollack et al., 1994). AACT activity was determined by incorporation of [^{14}C]acetyl-CoA into heat-stable and acid-stable [^{14}C]HMG-CoA (Weber and Bach, 1994). This test is a modification of the method used for the assay of avian HMGS introduced by Clinkenbeard et al. (1975). In our method we use ^{14}C-labeled acetyl-CoA as the only substrate, which is then converted to acetoacetyl-CoA by action of AACT. Endogenous HMGS uses this intermediate for the condensation with an additional acetyl-CoA unit to yield *S*-HMG-CoA. After stopping the reaction by adding HCl, followed by heating of the samples to 110°C, the thioesters were cleaved. Unreacted substrate (in the form of acetate or acetoacetate converted into acetone) evaporated, but HMG acid remained. Hence, the incorporation of radioactivity into a heat-stable and an acid-stable product indicated the combined activity of AACT and HMGS. Protein was quantified by the Lowry method (Bensadoun and Weinstein, 1976) with some modifications (Bach et al., 1986).

RESULTS

Generation of a Suitable Yeast *erg10* Mutant and Phenotype of Transformants

The original ergosterol-auxotrophic yeast strain F2sp5 (Servouse et al., 1984) is a derivative of the strain FL100,

which bears a mutation in the *GAL2* gene encoding Gal transferase. Thus, for heterologous expression of plant cDNAs placed under the *GAL1* promoter, we had to generate an *erg10A* recipient strain with the wild-type allele *GAL2* being reconstituted. This was achieved by crossing the two haploid strains F2sp5 (Mat **a**) and W303–1B (Mat α; Thomas and Rothstein, 1989). The new strain thus generated, termed KV5, was capable of growing at the permissive temperature of 28°C on Glc or Gal as a carbon source, but did not grow on Gal at 37°C (inducing conditions; Fig. 1). Both ergosterol-auxotrophic strains, F2sp5 and its derivative KV5, exhibited rather low transformation efficiencies, with approximately 1.3×10^3 Ura3$^+$-transformants/μg DNA. Transformation assays using electroporation (Becker and Guarente, 1991) were unsuccessful (data not shown). The problem of low transformation rates and/or fragility of cells has also been seen with other yeast *erg* mutants deficient in mevalonate kinase (*erg12–1*, Riou et al., 1994) or deficient in Δ^7-sterol-C5-desaturase (*erg3*, Gachotte et al., 1996). From a total of about 10^5 Ura3$^+$-transformants, 127 clones were found to be thermoresistant, but only 6 of them showed the wild-type phenotype that was dependent on the selected carbon source. These clones, as well as the transformed strain KV10 (see Fig. 1), grew at 37°C in the presence of Gal, but not when Glc was the sole carbon source. A parental strain, W303–1B (Thomas and Rothstein, 1989), was used as an Erg10$^+$ control; this grew under all conditions applied (Fig. 1). Although the *erg10* strain KV5 exhibited some residual growth on Glc at 37°C, it did not develop on Gal at this temperature.

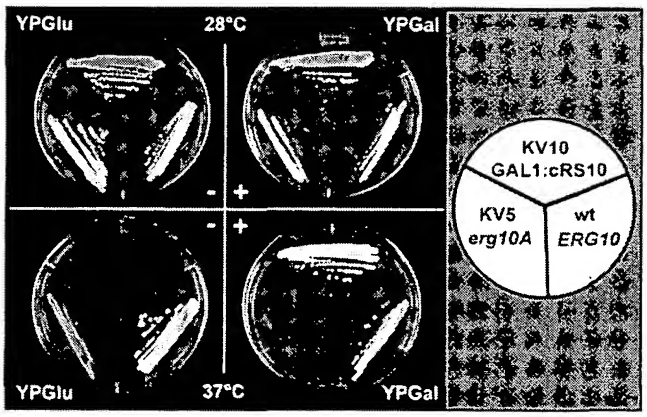

Figure 1. Culture of *erg10A* mutants and transformants expressing radish AACT and wild-type yeast cells. Cells were grown for 3 d at the permissive temperature of 28°C and at the nonpermissive temperature of 37°C on 2% Glc (YPGlu, noninducing conditions) and on 2% Gal (YPGal, inducing conditions), respectively. In each case, the medium contained 80 μg/mL ergosterol. Top, Strain KV10 (*GAL1*:cRS10 (*erg10A* [pYRS10]). Left, KV5 (*erg10A* [pYEUra3], plasmid without cDNA insert as a control). Right, Wild-type (wt) strain W303–1B (*ERG10*). Note that strain KV10 cannot grow at 37°C in the absence of Gal, whereas some growth of yeast cells with strain KV5 was observed at 37°C on Glc but not on Gal.

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          348                                                        *
Sccaact   P...SKVNVY GGAVALGHPL GCSGARVVVT LLSILQQEGG K..IGVAAIC
Sucaact   P...SKVNVY GGAVALGHPL GCSGARVVVT LLSILQQEGG K..IGVAAIC
Ctpaact   L...EKLNVY GGAVAMGHPL GCSGARIIVT LLSVLTQEGG R..FGVAGVC
Rscaact   P...EKVNVN GGAVSLGHPL GCSGARILIT LLGILKKRNG K..YGVGGVC
Hsmaact   P...QKVNIN GGAVSLGHPI GMSGARIVGH LTHALKQ..G E..YGLASIC
Hsmoact   I...SKTNVN GGAIALGHPL GGSGSRITAH LVHELRRRGG K..YAVGSAC
Rnmoact   P...SKTNVS GGAIALGHPL GGSGSRITAH LVHELRRRGG K..YAVGSAC
Aecoact   T...SKVNVN GGAIAIGHPI GASGCRILVT LLHEMKRRDA K..KGLASLC
Cacaact   M...NKVNVN GGAIALGHPI GASGARILVT LVHAMQKRDA K..KGLATLC
Zrcaact   P...SIVNVN GGAIAIGHPI GASGARILNT LLFEMKRRGA R..KGLATLC
Hscaact   P...EKVNIE GGAIALGHPL GASGCRILVT LLHTLERMGR S..RGVAALC
Ctpoact   E...EKLNIN GGAIALGHPL GETGARQYAT TIPLLKPG.. Q..IGLTSMC
Ylpoact   E...SKVNPR GGAIAIGHPL GATGARQFAT LLSELKESGK K..VGVTSMC
Hspoact   P...EKVNPL GGAVALGHPL GCTGARQVIT LLNELKRRGK RA.YGVVSMC
Rnpoact   A...EKVNPL GGAIALGHPL GCTGARQVVT LLNELKRRGT RA.YGVVSMC
Cspoact   P...EKINVN GGAIAIGHPL GATGARCVAT LLHEMKRRGK DCRPGVISMC
Eccoact   EQIDEKINLN GGAIALGHPL GCSGARISTT LLNLMERKDV Q..PGLATMC

          393
Sccaact   NGGGGASSIV IEKI..... ..........  ........
Sucaact   NGGGGASSVV IEKL..... ..........  ........
Ctpaact   NGGGGASAVV IEKIDADAKL ..........  ........
Rscaact   NGGGGASALV LEVV..... ..........  ........
Hsmaact   NGGGGASAML IQKL..... ..........  ........
Hsmoact   IGGGQGIAVI IQSTA..... ..........  ........
Rnmoact   IGGGQGISLI IQNTA..... ..........  ........
Aecoact   IGGGMGVALA VERK..... ..........  ........
Cacaact   IGGGQGTAIL LEKC..... ..........  ........
Zrcaact   IGGGMGVAMC IESL..... ..........  ........
Hscaact   IGGGMGIAMC VQRE..... ..........  ........
Ctpoact   IGSGMGSASI LVRE..... ..........  ........
Ylpoact   IGTGMGAASL VVAE..... ..........  ........
Hspoact   IGTGMGAAAV FEYPGN.... ..........  ........
Rnpoact   IGTGMGAAAV FEYPGN.... ..........  ........
Cspoact   IGTGMGAAAV FERGDCVDEL CNAKKVEGGV NLLSKDAR
Eccoact   IGLGQGIATV FERV..... ..........  ........
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Figure 2. Sequence homologies between thiolases at the C-terminal end. Amino acid residues that are common to all sequences are in boldface. Only full-length sequences have been considered. Using the DNASIS/PROSIS software from Hitachi, the nucleic acid sequence of cRS10 and the deduced amino acid sequence were used to search database libraries for homologies. Additional sequence analyses were performed with the BLAST network service, and sequence alignments were carried out with the PileUp program of the Genetics Computer Group sequence analysis software package. The numbering of amino acid residues corresponds to the radish AACT. The catalytically important Cys residue in this domain is marked by an asterisk (*). Putative subcellular localization: c, cytosolic; m, mitochondrial; p, peroxisomal. Database accession numbers are given in parentheses. Ae, *Alcaligenes eutrophus* (J04987); Ca, *Clostridium acetobutylicum* (U08465); Cs, *Cucumis sativus* (X67696); Ct, *Candida tropicalis* (AACT, D13471; OACT, D17321); Ec, *Escherichia coli* (fadA gene, J05498); Hs, *Homo sapiens* (AACT, mitochondrial, D90228 and M61117; OACT, peroxisomal, X12966); Rn, *Rattus norvegicus* (AACT, mitochondrial, D00512; OACT, mitochondrial, X05341, and peroxisomal, D90063 and J05269); **Rs**, *Raphanus sativus* (X78116); Sc, *Saccharomyces cerevisiae* (L20428); Su, *Saccharomyces uvarum* (X07976); Yl, *Yarrowia lipolytica* (X69988); Zr, *Zoogloea ramigera* (J02631).

Sequence Analysis

Sequence analysis of the 1462-kb insert of plasmid pYRS10 (EMBL accession no. X78116), which confers ergosterol autotrophy and thermoresistance to the yeast strain KV5 (*erg10*), revealed the existence of an open reading frame containing the entire coding unit for a protein of 406 amino acids (M_r 42,032). There was no evidence of the presence of a leader peptide (Von Heijne et al., 1989; De Hoop and Ab, 1992), as in the peroxisomal OACT from cucumber (462 amino acids, M_r 48,650). The precursor form of peroxisomal OACT was approximately 4 kD larger than the 45-kD subunit of the mature enzyme (Preisig-Müller and Kindl, 1993). At the amino acid level there exist significant sequence homologies (Fig. 2) (see Igual et al., 1992;

Kanayama et al., 1994; Mathieu et al., 1994; for additional refs. consult the accession numbers indicated in legend to Fig. 2) with domains of thiolases from mammals; from the yeasts *Saccharomyces uvarum*, *S. cerevisiae*, *Candida tropicalis*, and *Yarrowia lipolytica*; and from bacteria such as *Escherichia coli*, *Clostridium acetobutylicum*, and *Zoogloea ramigera* (Peoples et al., 1987). Homotetrameric (biosynthetic) thiolase from *Z. ramigera* has subunits of M_r 40,598, in complete agreement with the value determined by SDS-PAGE (Davis et al., 1987).

There are two conserved Cys residues that have been recognized as being important for *Z. ramigera* thiolase activity (Thompson et al., 1989; Williams et al., 1992). One is located in the N-terminal section of the enzymes (presumably Cys^{91} for radish AACT) and is involved in the formation of an acyl-enzyme intermediate already identified by Gehring and Harris (1970) using pig heart AACT. The other, located at the C-terminal extremity (corresponding to Cys^{390} in radish AACT; see Fig. 2), is the active site base involved in deprotonation in the condensation reaction (see Thompson et al., 1989; Williams et al., 1992; Mathieu et al., 1994, for discussion and further literature citations). The hydropathy profile of the deduced amino acid sequence of radish AACT, calculated according to Kyte and Doolittle (1982), exhibits the typical features of a soluble protein with internal hydrophobic core domains (not shown). The highly conserved Gly-rich domain in the C-terminal region (see Fig. 2) with the consensus sequence $\underline{G}_{355}$-$\underline{G}$-$\underline{A}$-I/$\underline{V}$-$\underline{S}$/V-$\underline{L}$/I-$\underline{G}$-$\underline{H}$-$\underline{P}$-I/$\underline{L}$-$\underline{G}$-X-$\underline{S}$/T-$\underline{G}$-X-$\underline{R}_{370}$ (underlined and index numbers indicate the radish sequence) seems to be important for the stability of the thiolase protein (Fukao et al., 1991). Within this domain (radish AACT: L_{360}-G-H-P-L-G-C_{366}) we found a high similarity to a heptapeptide (five identical residues, two conservative exchanges) of human lecithin-cholesterol acyltransferase (EC 2.3.1.43), which has been discussed as forming an interfacial lipid binding site (McLean et al., 1986).

Genomic DNA Gel Blot Hybridization Analysis

Genomic DNA gel blot hybridization (Fig. 3) with the radish cDNA (cRS10) suggests the presence of a single gene or a small family of genes. Only two bands were visible in the lanes containing *Bam*HI- and *Hin*dIII-digested DNA when hybridized under stringent conditions to the full-length cDNA probe. Because the cDNA sequence contains one internal restriction site each for *Bam*HI and for *Hin*dIII, and assuming that such restriction sites are not preserved in isogenes, this observation suggests a single-copy AACT gene in radish. Numerous bands in lanes in which DNA was digested with *Eco*RI, *Kpn*I, or *Xba*I and no internal restriction sites were present in the cDNA sequence most likely point to the occurrence of several introns within the radish AACT gene. When hybridizations were performed under less-stringent conditions, however, more signals could be detected in all lanes (not shown). This would be expected in view of the presence of strongly conserved regions in all AACT and OACT sequences isolated so far (see Kanayama et al., 1994; Mathieu et al., 1994). However, at this stage we would not exclude the presence of related genes encoding isozymes with differential intracellular localization and biochemical function.

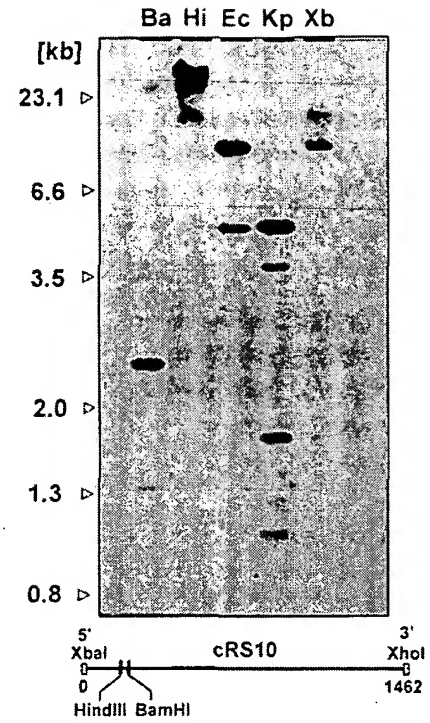

Figure 3. Gel blot analysis of genomic radish DNA. Genomic DNA (30 μg each) was digested with *Bam*HI (Ba), *Hin*dIII (Hi), *Eco*RI (Ec), *Kpn*I (Kp), and *Xba*I (Xb). Electrophoresis was done in 0.8% agarose gels. As a probe the complete 1.46-kb *Xba*I/*Xho*I cDNA fragment of cRS10 was used, previously DIG-11-UTP-labeled by the random priming method.

Heterologous Expression in Yeast

Gel blot analysis of RNA isolated from yeast transformant strain KV10 and parental strain KV5, transformed with the plasmid without the cDNA insert (Fig. 4), reveals a strong overexpression of radish cDNA cRS10 at the transcriptional level and its dependence on Gal induction (Fig. 4, A and B). Exposure of the film for 2 h led to the masking of a distinct band and to a positive signal over the whole separation range of the agarose gel. After exposure for only 20 min, a prominent band of about 1.5 kb appeared, with total RNA isolated from KV10 exclusively under inducing conditions (Fig. 4B). After longer exposure an additional transcript of approximately 1.2 kb became visible with RNA isolated from KV10 under *GAL1* promoter-repressing conditions, namely in the presence of Glc (Johnston and Davis, 1984). Since it could not be detected in the lane with RNA from parental strain KV5 transformed with the vector pYEUra3, a further transcript must have been read from cRS10, which, however, was not controlled by the *GAL1* promoter.

Measurement of Enzyme Activity in Yeast

For the assay of AACT in cell-free yeast extracts, we adopted the method of Weber and Bach (1994), which

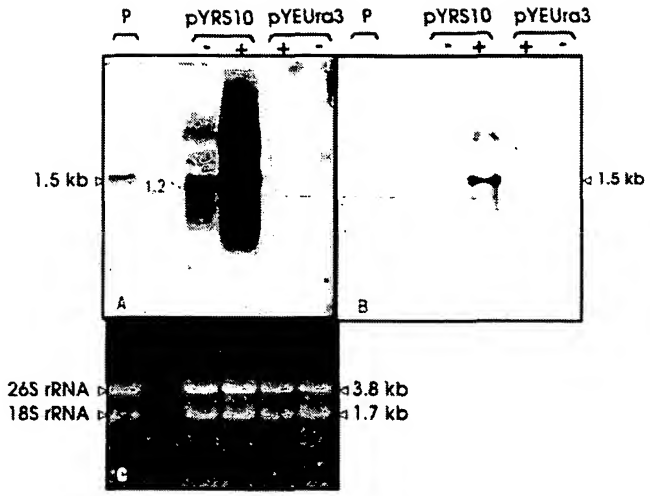

Figure 4. Transcription of radish cDNA cRS10 in yeast transformant KV10. Gel blot analysis of total RNA (10 μg) from yeast cells grown under inducing (+, 2% Gal) and noninducing (−, 2% Glc) conditions. KV10, (*erg10A* [pYRS10]) containing the radish AACT coding unit; KV5p, (*erg10A* [pYEUra3]) plasmid without insert as a control; P, RNA (10 μg) from 4-d-old etiolated radish seedlings for comparison; A, Exposition time 3 h; B, exposition time 20 min; C, ethidium bromide staining of the 1.2% agarose gel after electrophoresis (rRNA bands are of similar intensity). As a probe the complete 1.46-kb *Xbal/Xhol* cDNA fragment of cRS10 was used, which was DIG-11-UTP-labeled by the random priming method.

determines the combined activity of AACT and HMGS. When the yeast strain KV10 carrying the plasmid with the gene encoding radish AACT was grown on Glc minimal medium (supplemented with 4 mg/L ergosterol in liquid broth) at the permissive temperature of 26°C, there was practically no AACT activity measurable in total cellular protein extracts prepared using a detergent (Basson et al., 1986) (Fig. 5). As expected, wild-type yeast showed a similar AACT/HMGS activity when grown on either a Glc or a Gal medium. In strain KV10 expression of radish AACT led to an 8- to 10-fold increase of apparent AACT/HMGS activity over that of the wild type (Fig. 5). In the presence of 1233A (also called F244 or L659,699), a potent inhibitor of HMGS (Greenspan et al., 1987; Tomoda et al., 1987), this activity was completely blocked (data not shown). The ergosterol-auxotrophic strain used for transformation apparently was slightly leaky, with about 20% of wild-type activity, as was shown by Dequin et al. (1988b) using strain F2sp5 (*erg10A*). Addition of PQQ and Fe(II), cofactors that stimulated the AACT/HMGS enzyme system solubilized from radish membranes (Weber and Bach, 1994), had no effect (Fig. 5). When yeast cell homogenates were subfractionated and assayed for AACT/HMGS, it was clearly revealed that specific enzyme activity was associated with the cytosolic fraction, but not with resuspended membrane pellets (Fig. 6). This is in clear contrast to the intracellular localization of two intact radish HMGR isozymes expressed in a mevalonate-auxotrophic yeast mutant originally generated by Basson et al. (1988), in which enzyme activity was exclusively associated with membranes (Vollack et al., 1994). The degree of overexpression in measurable enzyme activity was not as high as expected from the RNA blot analysis, but was still about 10 times higher than the values found in protein extracts from wild-type yeast. Similar values have been found in yeast cells by overexpression of the *ERG10* gene from *S. uvarum* cloned on a multi-copy 2-μm plasmid (Dequin et al., 1988a).

Expression of AACT in Radish

RNA gel blot analysis of total RNA isolated from etiolated, intact radish seedlings (from d 2–8 after germination) indicated the presence of a 1.5-kb transcript, with little or no change in intensity (Fig. 7). In comparison, the same RNA samples were hybridized to two probes specifically recognizing radish *HMG1* (EMBL accession no. X68651, nucleotides 500–521) and *HMG2* (EMBL accession no. X68652, nucleotides 464–484). (The nomenclature we used for the characterization of HMGR-encoding genes in radish

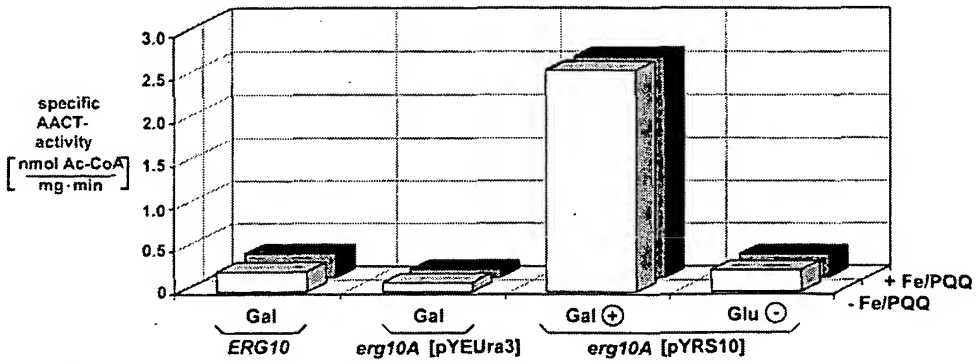

Figure 5. AACT activity in total protein extracts from yeast: strain KV10 (*erg10A* [pYRS10]); strain KV5p (*erg10A* [pYEUra3]); and strain W303-1B (ERG10). Activity was measured by incorporation of [1-^{14}C]acetyl-CoA into heat-stable and acid-stable HMG acid via HMG-CoA (see Weber and Bach, 1994, for details). Medium: YNB (minimal medium; 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 50 μg/mL His, adenine, Len, 4 μg/mL ergosterol) + His + adenine + Leu, 4 μg/mL ergosterol. Gal, 2% Gal; Glu, 2% Glc; temperature, 28°C. The tests were also performed in the presence of 25 μM PQQ plus 50 μM Fe(II), conditions that were described as stimulating the in vitro activity of the coupled enzyme system AACT/HMGS in extracts from radish membranes. Note that no difference is visible.

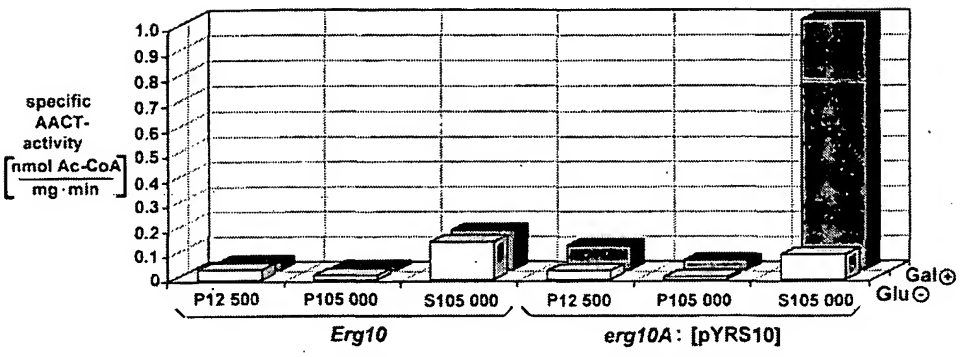

Figure 6. AACT activity in subcellular fractions isolated from transformed yeast. Homogenates were prepared from strain KV10 (*erg10A* [pYRS10]) and from wild-type strain W303–1B. Subcellular fractions were isolated by means of differential centrifugation at 12,500*g* (organelle fraction) and at 105,000*g* (microsomes). Soluble proteins were found in the following supernatant (S105 000). Medium: YNB [minimal medium; 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 50 μg/mL His, adenine, Leu, 4 μg/mL ergosterol] + His + adenine + Leu, 4 μg/mL ergosterol. Gal, 2% Gal; Glu, 2% Glc. Temperature during culture was 28°C (permissive conditions).

depended on the sequence of their cloning). *HMG2* is expressed to a considerable degree from d 2 to 8 of germination, whereas the signals for *HMG1* were much weaker. When we examined gene expression in different parts of 6-d-old etiolated seedlings, the 1.5-kb signal was found with RNA isolated from roots, stems, and, to some extent, from cotyledons of radish seedlings, suggesting a more or less constitutive expression of the corresponding AACT gene in all major parts (Fig. 8A). However, the signal intensity was higher with RNA isolated from cotyledons of 6-d-old light-grown seedlings than from etiolated seedlings (Fig. 8B). In a further experiment, seedlings were grown for 6 d in the light in the presence of 10 μM mevinolin, a specific inhibitor of HMGR and, hence, of de novo phytosterol biosynthesis (Bach and Lichtenthaler, 1983). In the RNA gel blot we noted the appearance of an additional transcript of about 800 bp with RNA isolated from hypocotyls and to some extent with RNA from cotyledons (Fig. 8C)

When we looked for a possible explanation for the appearance of another transcript from cRS10 we noted that on the noncoding strand we could identify putative TATA box-like elements that could give rise to the formation of antisense transcripts (the numbering corresponds to positions on the coding strand): (1) $T_{1273}ATAAAT_{1267}$; (2) $T_{1299}ATATAG_{1293}$; (3) $T_{1345}ATCAAA_{1339}$; and (4) $T_{1416}ATAATA_{1410}$.

Therefore, we used strand-specific DIG-labeled RNA probes for the detection of putative sense and antisense

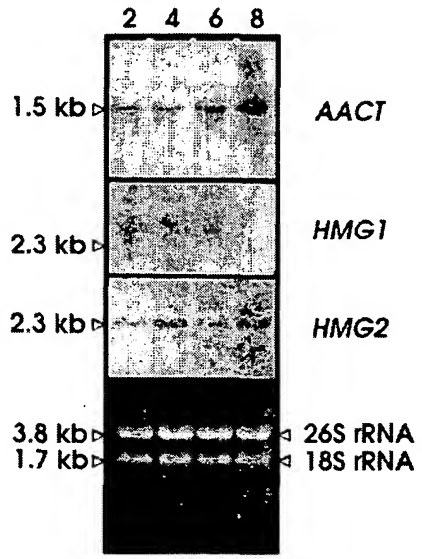

Figure 7. AACT gene expression in etiolated radish seedlings during germination. For RNA gel blot analysis, total RNA was isolated from seedlings from d 2 to 8 after germination in darkness. Ten micrograms of RNA each were hybridized to the 1.4-kb cDNA cRS10, which was labeled with DIG-11-dUTP by random priming. For comparison, the same RNA samples were hybridized to a 22-mer specifically recognizing radish *HMG1* and to a 21-mer recognizing *HMG2*. Oligonucleotides were end-labeled with DIG-11-dUTP. Bottom, Ethidium bromide-stained 1.2% agarose gel with rRNA bands.

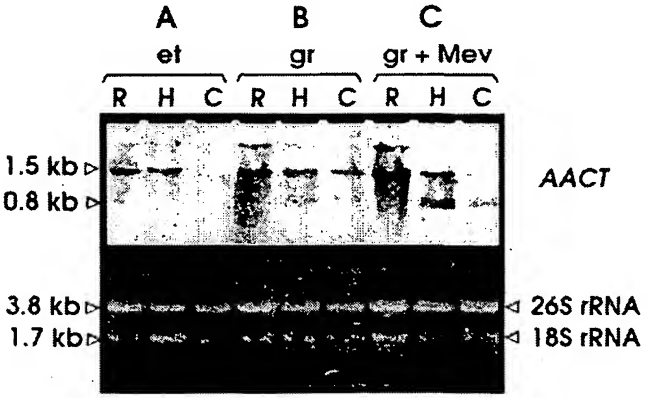

Figure 8. Tissue-dependent expression of radish AACT. RNA gel blot analysis of total RNA (10 μg each) isolated from roots (R), hypocotyls (H), and cotyledons (C) of 6-d-old radish seedlings cultivated in darkness (A, et), in light (B, gr), or in light in the presence of 10 μM mevinolin (C, gr + Mev). The light/dark cycles were 14 and 10 h, respectively. Light intensity was 500 μmol m^{-2} s^{-1}. Room temperature (25°C) and humidity (80%) were kept constant. Hybridization was as in Figure 7.

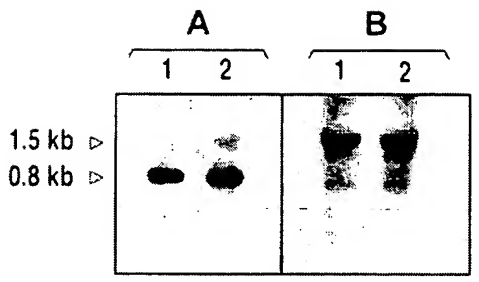

Figure 9. Sense- and antisense-specific detection of mRNA isolated from 6-d-old light-grown radish seedlings. Seedlings were grown on water (lanes 1, control) or on water in the presence of 10 μM mevinolin (lanes 2). The light and culture conditions were as in Figure 8. Antisense (A) and sense mRNA species (B) were detected with DIG-UTP-labeled, single-strand-specific RNA probes using pcRS10 (derivative of pBluescript SK) as the cDNA template. T3 RNA polymerase generated the antisense-specific RNA probe, and T7 RNA polymerase generated the sense-specific probe.

transcripts in radish (Fig. 9), using 6-d-old light-grown radish seedlings cultivated on water (control) and on 10 μM mevinolin. The antisense-specific probe (Fig. 9A) specifically hybridized to a band of approximately 0.8 kb. The signal arising from RNA isolated from mevinolin-treated light-grown seedlings (Fig. 9A, lane 2) was slightly more intense. The sense-specific probe (Fig. 9B) turned out to hybridize to the 1.5-kb transcript. The use of DIG-labeled RNA probes resulted in a sensitivity of RNA detection that was at least 10 times higher than that resulting from DIG-labeled cDNA probes. Some weak signals in experiment B might have resulted because total RNA was used and in some unspecific hybridization occurred.

DISCUSSION

Several lines of evidence indicate that we have successfully cloned a cDNA containing the entire coding unit for a radish AACT, resulting in a protein of M_r 42,032. There are significant sequence homologies to other eukaryotic and prokaryotic thiolase genes, but clearly more are related to AACT than to OACT encoding genes. A strong argument is the functional expression in yeast, and thereby complementation of the *erg10* mutation. Activity, determined as conversion of acetyl-CoA to HMG-CoA in the presence of AACT and endogenous HMGS, can be found only in the soluble fraction but not in membrane pellets isolated from yeast transformants. This and the complete lack of a typical transit peptide characteristic of glyoxysomal OACT recently cloned from cucumber (Preisig-Müller and Kindl, 1993), which the radish gene resembles significantly, but much less than cytosolic AACT from *S. uvarum* (Dequin et al., 1988b) or from *S. cerevisiae* (Hiser et al., 1994), led us to conclude that we have indeed cloned the first plant cDNA encoding cytosolic (biosynthetic) AACT.

Recently, a membrane-associated enzyme system was purified from radish, catalyzing the conversion of acetyl-CoA into a heat-stable and acid-stable product considered to contain HMG-CoA, which was thus referred to as AACT/HMGS. Its apparent in vitro activity is greatly stimulated in the presence of Fe(II) and a quinone cofactor (Weber and Bach, 1994). However, when we used the same assay conditions, namely determination of [2-^{14}C]acetyl-CoA incorporation in the presence of Fe(II) and PQQ as the most efficient quinone cofactor found so far, we could not observe any significant stimulation of enzyme activity with cell-free extracts of transformed yeast. From these observations we conclude that the product of the cloned gene is not identical to the membrane-associated AACT/HMGS system described before (Weber and Bach, 1994). Cloning of this radish AACT gene and of an Arabidopsis HMGS gene (Montamat et al., 1996) provides convincing evidence for the presence of separate AACT and HMGS enzymes in plant cells, although they seem to behave similarly during various purification steps (Alam et al., 1991; Van der Heijden et al., 1994).

Recently, Mathieu et al. (1994) published the crystal structure of peroxisomal OACT from *S. cerevisiae* refined at 2.8-Å resolution. Apparently, the homodimeric unliganded yeast OACT comprises three domains: two compact core domains that have the same fold and a loop domain. Each monomer contains an identical core domain: that is folded into a mixed, five-stranded β-sheet covered by helices on each side and assembled into a five-layered $\alpha\beta\alpha\beta\alpha$ structure. Although peroxisomal OACT proteins isolated from seven organisms form a much closer family (152 identical residues out of 417) than the 40 identical residues found in all 21 thiolase sequences in databases (Mathieu et al., 1994), it is reasonable to assume that the three-dimensional structures, especially around the pocket of the active center shaped by highly conserved residues close to the C-terminal end, are being conserved in the same way. However, a final answer awaits the crystallization and x-ray analyses of plant AACT protein.

Gal-induced expression of radish AACT in the yeast *erg10* mutant reestablishes the wild-type phenotype, namely growth at 37°C and ergosterol autotrophy. Repression by Glc of the *GAL1* promoter results in the phenotype of the conditionally lethal *erg10* mutation. A surprising characteristic of strain KV10 (carrying pYRS10) when it was cultivated on Glc medium at 37°C with a high selection pressure was the complete absence of so-called "revertants," which are typical of the *erg10A* mutant (strain KV5; see also Dequin et al., 1988b). The revertants were observed more rarely in cells grown at the restrictive temperature of 37°C on a Gal medium than on a Glc medium. This might be explained by the fact that the cells used for these experiments had originally been taken from Glc plates and, as a consequence, had adapted to Glc as a carbon source. Even under restrictive "heat stress" at 37°C, the freshly plated Glc-adapted *erg10A* cells were able to convert Glc into energy, whereas on Gal an energy-consuming adaptation to the Gal metabolism was required. This lack of energy, together with the blocking of the isoprenoid biosynthesis, would lead to an immediate growth arrest on Gal, whereas on Glc some additional cell divisions would be possible, with a certain probability of back mutation.

Gel blot analysis of RNA isolated from pYRS10 transformants in which the *GAL1* promoter was induced showed a strong signal for a 1.5-kb transcript, which does not appear

in RNA isolated from control yeast cells (strain KV5 carrying the parental plasmid pYEUra3). However, under Glc-repressed conditions (Johnston and Davis, 1984) we noted the appearance of a smaller hybridizing transcript (about 1.2 kb). Since no signal was obtained with control cells, the only conclusion that could be drawn was that this transcript arose from a transcript of the radish cDNA cRS10, which had not been controlled by Glc repression of the *GAL1* promoter. Analysis of the nucleotide sequence of cRS10 in the downstream, nontranslated region showed the presence of TA-rich nucleotide stretches comprising several TATA-box elements on the complementary strand. In particular, the first TATA box (positions 1273–1267) corresponds exactly to the consensus sequence, which determines the transcription starting point in yeast (Hahn et al., 1985) and in other eukaryotes (Dynan and Tijan, 1985). If only one of the putative TATA elements on the antisense strand were recognized by the yeast's transcription initiation factors and, thus, the transcription of an antisense mRNA were initiated, this could lead to the hybridization with rather well-conserved transcripts of the mutated but still transcribed yeast *erg10A* allele. For yeast cells, the result would be something like a gene disruption.

In terms of measurable enzyme activity, we arrived at an overexpression of AACT about 8 to 10 times higher than the wild-type values. This observation corresponds well with that made by Dequin et al. (1988a), who overexpressed AACT from *S. uvarum* in a yeast *erg10* strain. These authors used an assay system that measures thiolase activity exclusively in the cleavage direction, which is thermodynamically favored. In our coupled AACT/HMGS assay, in which the HMGS has to arise from the yeast HMGS gene, such an apparent increase in enzyme activity can be interpreted in such a way, at least in *S. cerevisiae*, that AACT appears to catalyze the rate-limiting step for the two-step conversion of acetyl-CoA to HMG-CoA.

Overexpression of cytosolic AACT in yeast has no apparent negative effect on the growth behavior of its cells. This has already been shown with yeast overexpressing the AACT gene isolated from *S. uvarum* (Dequin et al., 1988a) or for yeast overexpressing other enzymes downstream on the sterol pathway, mevalonate kinase (Riou et al., 1994) and phosphomevalonate kinase (Tsay and Robinson, 1991). From the results obtained by Dequin et al. (1988a) it seems clear that overexpression of AACT does not significantly affect the sterol pattern nor its total content. Thus, other enzymatic steps downstream on the pathway must be responsible for substrate flow regulation from acetyl-CoA to ergosterol. On the other hand, a complete knockout of the yeast *ERG10* gene is lethal (Servouse et al., 1984; Hiser et al., 1994). Only thermolabile *erg10* mutants could be isolated; these grow at the permissive temperature of 26°C but not at 36°C (Servouse et al., 1984). It is noteworthy that the original *erg10* mutant exhibits about 20% of wild-type AACT activity (Dequin et al., 1988b), which indicates that the gene is not completely inactive. The product of the mutated gene could therefore exhibit a lower catalytic efficiency and/or a higher thermosensibility. Such an amino acid exchange in the catalytic center has been demonstrated for the *erg20* mutation of yeast farnesyl pyrophosphate synthase (Blanchard and Karst, 1993). For instance, the difference in the heat stability of the sweet protein mabinlin is a result of the replacement of a single amino acid residue (Nirasawa et al., 1994). The lethality of a complete lack of AACT might indicate a further role of this enzyme independent of its inclusion in the sterol pathway, e.g. in the biosynthesis of pre-squalene products that are essential for cell-cycle regulation.

Recently, an AU-rich RNA binding protein has been isolated from the flesh fly *Sarcophaga peregrina*, and this protein has subsequently been identified as a thiolase (Nanbu et al., 1993). The partial amino acid sequences of two peptides obtained from the 39-kD protein showed high similarities to rat and yeast OACT and in fact exhibited thiolase activity. Since rat mitochondrial OACT showed affinity to the AU-rich RNA, this RNA-binding activity might be an intrinsic character of thiolase (Nanbu et al., 1993). It is tempting to assume that in this way thiolase protein could somehow down-regulate its own synthesis once sufficient amounts have been formed. The presence of AU-rich regions in the untranslated, downstream region of our cDNA clone could possibly match such a requirement.

Especially when radish seedlings were grown in the presence of the HMGR inhibitor mevinolin, RNA blot analysis using DIG-labeled cDNA revealed the appearance of a hybridizing band of approximately 800 bp. Under such conditions, namely blockage of de novo phytosterol biosynthesis (Bach and Lichtenthaler, 1983), this might indicate some stress response of the plant that remains to be further characterized. This additional 800-bp band had already been barely visible with RNA isolated from control seedlings. It was initially interpreted as demonstrating the presence of a gene bearing considerable sequence homology to the AACT gene. However, this could be ruled out in view of the results from the genomic DNA gel blot analysis. It is also possible that we might have obtained an additional transcript that arises from alternative splicing. The above-mentioned TATA-box elements in the complementary strand might also give rise to another transcript. In a preliminary experiment, we were able to demonstrate the presence of a putative antisense transcript by strand-specific hybridization to DIG-labeled RNA. Synthesis of an AACT antisense mRNA would provide the means for a down-regulation of AACT activity, which could be rapidly reversed due to the small lifetime of RNA-RNA duplex hybrids (see Inouye, 1989). Such a mechanism, including the synthesis of antisense mRNA for in vivo expression, was described for α-amylase from barley (Rogers, 1988; see also Mol et al., 1990). However, additional research, including characterization of corresponding genomic clones and high-resolution mapping of putative sense and antisense RNAs, is needed to clarify these questions.

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Poly-β-hydroxybutyrate (PHB) Biosynthesis in *Alcaligenes eutrophus* H16

IDENTIFICATION AND CHARACTERIZATION OF THE PHB POLYMERASE GENE (*phbC*)*

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The *phbC* gene encoding the third enzyme of the poly-β-hydroxybutyrate biosynthetic pathway, poly-β-hydroxybutyrate polymerase, in *Alcaligenes eutrophus* H16 has been identified by the complementation of poly-β-hydroxybutyrate negative mutants of *A. eutrophus* H16. These results demonstrate that the three enzymes of the poly-β-hydroxybutyrate biosynthetic pathway are organized *phbC-phbA-phbB*. Expression of all three genes in *Escherichia coli* results in a significant level (50% dry cell weight) of poly-β-hydroxybutyrate production. *phbC* encodes a polypeptide of M_r = 63,900 which has a hydropathy profile distinct from typical membrane proteins indicating that poly-β-hydroxybutyrate biosynthesis probably does not involve a membrane complex.

A wide range of microorganisms accumulate poly-β-hydroxybutyrate (PHB),[1] the polymeric ester of D(−)-3-hydroxybutyrate, as an intracellular energy reserve material when grown under conditions of nutrient limitation (1). Both the level of accumulation and molecular weight of the PHB produced vary depending on the individual bacterial species. Recent fermentation studies of *Alcaligenes eutrophus* (2–5) and *Pseudomonas oleovarians* (6, 7) have identified a whole range of polyhydroxyalkanoate (PHA) storage polymers. The PHA polymers are heteropolymers of the D-isomer of β-hydroxyalkanoates with variation occurring predominantly in the length of the side chains (CH_3-C_8H_{17}). However, when grown in the presence of 5-chloropentanoic acid, *A. eutrophus* incorporates 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate into the polymer (4).

We have recently begun to investigate the biochemistry and molecular genetics of PHB biosynthesis in the chemolithoautrophic bacterium *A. eutrophus* H16. This organism has a remarkable capacity for accumulating PHB, *i.e.* up to 70–80% dry cell weight, under conditions of nitrogen or phosphate limitation (1). The enzymatic synthesis of the hydrophobic granules of PHB from acetyl-CoA has been studied in a number of bacteria and in most organisms proceeds via a three-enzyme pathway (8). Condensation of two acetyl-CoA units to form acetoacetyl-CoA is followed by the chiral reduction to D(−)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase and finally polymerization by PHB polymerase. In view of the incorporation of D(−)-hydroxyacyl-CoA substrates other then 3-hydroxybutyryl-CoA, it is useful to consider this pathway in two parts, *i.e.* (*a*) the synthesis of D(−)-hydroxyacyl-CoA substrates and (*b*) polymerization. Clearly, additional mechanisms are required to synthesize substrates for the polymerase other than C_4, C_5, and possibly C_6 units. For example, in *Rhodospirillum rubrum* L(+)-3-hydroxybutyryl-CoA is formed first and converted to the D(−)-isomer by the action of two enoyl-CoA hydratases.

In *Zoogloea ramigera* and *A. eutrophus* H16, the genes encoding β-ketothiolase (*phbA*) and NADP-specific acetoacetyl-CoA reductase (*phbB*) are closely linked and have been characterized (9–11) and used to overproduce the enzymes for mechanistic analysis. As PHB polymerase has proven difficult to purify from its native source (8), we are following the strategy of isolating the gene in order to facilitate the mechanistic analysis of this enzyme and investigate the regulation of PHB metabolism in *A. eutrophus*. Here we report the identification and characterization of the PHB polymerase gene *phbC* and demonstrate that the expression of all three genes of the pathway (*phbC-phbA-phbB*) in *E. coli* results in the accumulation of significant levels of PHB in this bacterium.

MATERIALS AND METHODS

Media and Culture Conditions—*E. coli* strains were grown in LB medium NaCl, 10 g/liter; Tryptone, 10 g/liter; yeast extract, 10 g/liter) or 2XTY medium (NaCl, 5 g/liter; Tryptone, 16 g/liter; yeast extract, 10 g/liter). For the production of PHB by *E. coli* containing recombinant plasmids, minimal media (12) was used, with the modification that the $(NH_4)_2SO_4$ concentration was decreased to 0.04%.

A. eutrophus strains were grown in Trypticase soy broth (TSB, BBL Microbiology Systems, Cockeysville, MD) or a defined minimal medium composed of 0.39 g/liter $MgSO_4$, 0.45 g/liter K_2SO_4, 12 ml of 1.1 M H_3PO_4, 15 mg/liter $FeSO_4 \cdot 7H_2O$; 24 ml of trace elements (20 mg/liter $CuSO_4 \cdot 5H_2O$; 100 mg/liter $ZnSO_4 \cdot 6H_2O$; 100 mg/liter $MnSO_4 \cdot 4H_2O$; 2.6 g/liter $CaCl_2 \cdot 2H_2O$). The pH was adjusted to 6.8 with NaOH and the medium sterilized by autoclaving. NH_4Cl was added to a final concentration of 0.1 or 0.01% as nitrogen source, and fructose was added to a final concentration of 0.5–1% (w/v).

DNA Manipulations—Restriction endonucleases, T4 DNA ligase, and DNA polymerase 1 were obtained from New England Biolabs and used under conditions provided by the manufacturer. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. All routine DNA manipulations, including plasmid purifications, *E. coli* transformations etc. were performed using methods described by Maniatis *et al.* (13). Chromosomal DNA was purified from *A. eutrophus* strains, grown to late logarithmic phase in TSB as described previously (14). Transfer of restriction-digested DNA samples from agarose gels to nitrocellulose filters, prehybridization, and hybridization with ^{32}P-labeled DNA probes has been described by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) JO5003.

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[1] The abbreviations used are: PHB, poly-β-hydroxybutyrate; PHA, polyhydroxyalkanoate; CoA, coenzyme A; kb, kilobases.

Peoples *et al.* (9). Rapid plasmid isolation from *A. eutrophus* recombinant strains, for restriction analysis, were performed by the alkaline extraction procedure (15).

Conjugation in A. eutrophus—The conjugal transfer of the broad host range plasmid, pLARF3, or recombinant derivatives of pLAFR3, into *A. eutrophus* was performed using the same method described by Easson *et al.* (14). In this case, however, the recipient *A. eutrophus* cells were not sonicated, and transconjugants were selected on *A. eutrophus* mineral agar plates containing 0.01% NH_4Cl as nitrogen source, 1% (w/v) fructose as carbon source, and 10 μg/ml tetracycline.

For Tn5 mutagenesis, a spontaneous streptomycin resistant strain of *A. eutrophus* 11599 (1599 S1) was used. Transfer of pRK602 (Tn5) was carried out as described above using *E. coli* MM294A (pRK602) as the only donor. *A. eutrophus* strains containing Tn5 were selected for by growth on streptomycin (500 μg/ml) and kanamycin (100 μg/ml).

Amplification and Identification of PHB-deficient Mutants—To identify PHB-deficient strains of *A. eutrophus*, we applied the amplification and screening procedures described by Schlegel and Oeding (16). A pool of around 10^5 Kan^R transconjugants (Tn5 insertion mutants) was inoculated into 10 ml of mineral media containing 0.01% NH_4Cl, 1% fructose, and 100 μg/ml kanamycin and incubated for 18 h at 30 °C. This culture was then used to inoculate 100 ml of the same medium and incubated for 30 h at 30 °C. To amplify PHB-deficient mutants, aliquots of this culture containing approximately 10^9 cells were fractionated on sucrose step gradients and plated out on mineral agar plates containing 0.01% NH_4Cl, 1% fructose, and 100 μg/ml kanamycin. After growth for 4–5 days at 30 °C, opaque (PHB-deficient) and white (PHB-containing) colonies were readily distinguished.

Analysis of Proteins—100-ml cultures of *A. eutrophus* strains were grown at 30 °C for 40 h in TSB with antibiotic selection as appropriate. Cells were harvested by centrifugation, resuspended in 2 ml of lysis buffer (10 mM Tris·Cl, pH 8.0; 5 mM β-mercaptoethanol; 5 mM EDTA, 0.02 mM phenylmethylsulfonyl fluoride; 10% v/v glycerol), and lysed by sonication. An aliquot of the lysate, cleared of cell debris by centrifugation, was used for β-ketothiolase and NADP-specific acetoacetyl-CoA reductase assays as previously described (10, 17). PHB polymerase assays were performed by measuring the incorporation of D-[^{3}H]hydroxybutyryl-CoA (specific activity ~2 μCi/μmol) as described by Fukui *et al.* (18). Protein concentrations were determined by the method of Bradford (19) using Bio-Rad assay solution and bovine serum albumin as the standard. *E. coli* maxi-cell labeling studies were performed as described by Sancar *et al.* (20).

PHB Purification and Quantitation—To determine the level of PHB in different strains, 100-μl aliquots of the crude lysates (described above) were treated with 1.2 ml of 5% sodium hypochlorite solution for 1 h at 37 °C. The insoluble PHB was then harvested by centrifugation for 10 min in a microcentrifuge, washed successively with 1 ml of H_2O, 1 ml acetone, 1 ml of ethanol, and dried under vacuum. PHB concentrations were then determined spectrophotometrically as described by Law and Slepecky (21) using a standard curve and expressed as milligrams of PHB/milligram of protein.

Plasmid Constructions and Complementation Analysis—Plasmids pLA29, pLA41, and pLA42 were constructed by cloning restriction fragments of the pAeT29 insert into the broad host range vector pLAFR3 for complementation analysis of the PHB negative *A. eutrophus* strains. pLAFR3 is a derivative of pLAFR1 (22) containing a pUC8 polylinker cloning site inserted into the *Eco*RI site.[2] Fig. 1*b* illustrates the origin of the different fragments of pAeT9 which were cloned into pLAFR3. pLA29 was constructed by ligating the entire 15-kb *Eco*RI insert from pAeT29 into the *Eco*RI site of pLAFR3. To facilitate the construction of pLA41 and pLA42, the corresponding fragments (Fig. 1*b*) were first cloned into pUC18 to produce plasmids pAeT41 and pAeT42. The fragments were then excised, by digestion with *Bam*HI and *Eco*RI, from the pUC18 plasmids, and ligated into *Bam*HI/*Hin*dIII-digested pLARF3. For the construction of pAeT41, the 5-kb *Sma*I-*Eco*RI fragment from pAeT29 was purified and ligated into *Sma*I/*Eco*RI-digested pUC18. Deletion of the 2.3-kb *Pst*I fragment containing the β-ketothiolase and acetoacetyl-CoA reductase structural genes by partial *Pst*I digestion of pAeT41 DNA and religation was used to construct pAeT42.

[2] B. Staskowicz, personal communication.

RESULTS

To identify and isolate the gene(s) encoding PHB polymerase, we used the strategy of constructing, characterizing, and complementing PHB negative mutants of a derivative (11599 S1, Table I) of *A. eutrophus* H16. Transposon mutagenesis allowed us to use DNA hybridization analysis to map the chromosomal location of the Tn5 insertion in any interesting strains. Potential PHB negative mutants were identified by their opaque colony phenotype when grown on nitrogen-limited minimal agar plates (see "Materials and Methods"). Preliminary DNA hybridization analysis using a Tn5 DNA probe identified three different classes of mutants (data not shown). Mutant strains PHB 2, PHB 3 and PHB 19, being representative of each class, were used in subsequent studies. The location of the chromosomal Tn5 insertion in each of the mutant strains with respect to the β-ketothiolase (*phb*A) and NADP-specific acetoacetyl-CoA reductase (*phb*B) genes, cloned in plasmids pAeT10 and pAeT29 (11), was mapped using these plasmids in a series of DNA hybridization experiments (data not shown). In the case of strain PHB 2 and strain PHB 3, the Tn5 insertion causing the opaque phenotype was located in the chromosome approximately 1.2 and 1.6 kb, respectively, upstream from the *phb*A-*phb*B genes as illustrated on Fig. 1*a*. For strain PHB 19, the Tn5 insertion was located elsewhere on the *A. eutrophus* chromosome.

The results of a biochemical analysis of wild type H16 and each of the mutant strains PHB 2, PHB 3, and PHB 19 is presented in Table II. 100-ml stationary phase cultures of each strain were harvested, lysed, and assayed for PHB content and β-ketothiolase, NADP-specific acetoacetyl-CoA reductase, and PHB polymerase activities. Under these growth conditions, the wild type H16 produces a significant level of PHB (1.3 mg PHB/mg protein, Table II) and has a high level of all three enzyme activities. Mutant strains PHB 2 and PHB 3 produce essentially no PHB, and strain PHB 19 produces only 5% of the wild type level (Table II). We were unable to detect PHB polymerase activity in any of these mutant strains, however, the presence of PHB in the lysate of strain PHB 19 indicates that the activity is there although probably at a level which the assay cannot detect. β-Ketothio-

TABLE I

Bacterial strains and plasmids

The following abbreviations were used: Strep, streptomycin; Tc, tetracycline; Nm, neomycin; Cm, chloramphenicol; Ap, ampicillin.

Strain	Relevant characteristics	Ref.
E. coli		
JM83		
DH5α	Host strain for plasmids	BRL
A. eutrophus		
H16	Wild type strain	ATCC17699
11599		NCIB 11599
11599S1	Strep^r	This Study
PHB 2	H16[*phb*2::Tn5]	This Study
PHB 3	H16[*phb*3::Tn5]	This Study
PHB 19	H16[*phb*19::Tn5]	This Study
Plasmids		
pAeT29	*phb*A-*phb*B	11
pAeT10	*phb*A-*phb*B	11
pLAFR3	Tc^r, cosmid vector	B. Staskawicz
pRK2013	Nm^r	
pRK602	Cm^r, Nm^r, pRK2013 nm::Tn9 containing Tn5	23
pUC18	Ap^r	24
pUC19	Ap^r	24

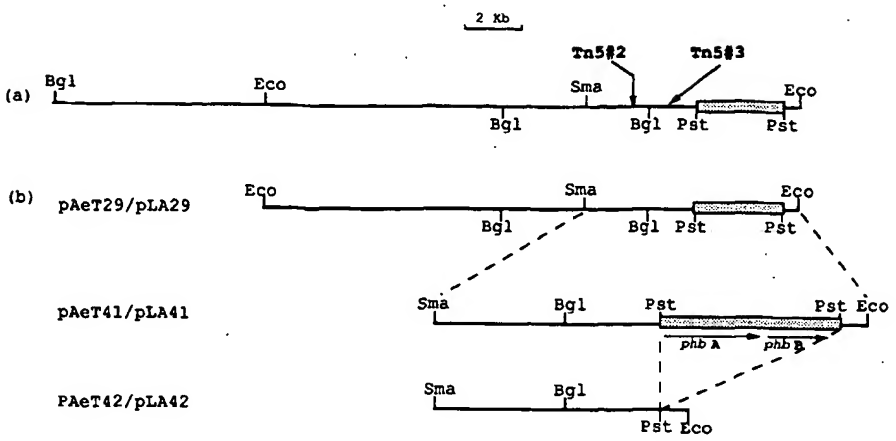

FIG. 1. **Restriction maps.** *a*, restriction map of the chromosomal region into which Tn5 has inserted in the *A. eutrophus* strains PHB 2 and PHB 3. The location of the *phb*A-*phb*B structural genes (11) are indicated by the *boxed region*. Restriction sites are as follows: *Bgl*, BglII; *Eco*, EcoRI; *Pst*, PstI; *Sal*, SalI; and *Sma*, SmaI. *b*, restriction maps of plasmid pAeT29 and subclones in the vectors pUC18 (pAeT10, pAeT41, and pAeT42) and pLAFR3 (pLA29, pLA41, and pLA42).

TABLE II

Analysis of PHB negative strains

Biochemical analysis of mutant and complemented *A. eutrophus* H16 strains. Results shown are the average of two or more experiments.

Strain	PHB[a]	Thiolase[b]	Reductase[b]	Polymerase[c]
H16	1.3	8.9	1.0	3.9×10^5
PHB 2	<0.01	4.0	0.5	ND[d]
PHB 3	<0.01	3.4	0.5	ND
PHB 19	0.06	3.2	0.4	ND
H16/pLA29	1.0	28.7	9.2	5.3×10^5
PHB 2/pLA29	1.5	27.5	3.5	3.8×10^5
PHB 3/pLA29	0.9	24.8	4.4	0.7×10^5
PHB 19/pLA29	1.8	26.7	4.9	1.0×10^5
PHB 2/pLA41		18.0	3.7	0.9×10^5
PHB 2/pLA42	1.2	2.0	0.3	4.3×10^5
PHB 3/pLA42	0.9	5.5	0.5	0.6×10^5
PHB 19/pLA42	1.2	5.5	0.4	0.6×10^5

[a] mg/mg of protein.
[b] units/mg of protein.
[c] cpm/min/mg of protein.
[d] ND, no detectable activity.

TABLE III

Expression of phbC-A-B in E. coli

Biochemical analysis of recombinant *E. coli* strains. Results shown are the average of two or more experiments.

Plasmid	Thiolase	Reductase	Polymerase	Poly(β)-hydroxybutyrate
	units/mg protein	*units/mg protein*	*cpm/min/mg protein*	*mg/mg protein*
pUC18	0.5	ND[a]	ND	0.015
pAeT41	59.0	2.5	2.4×10^4	2.977
pAeT42	0.9	ND	0.02×10^4	0.011

[a] ND, no detectable activity.

lase activities in all three mutants are reduced to the order of 45% (PHB 2) to 38% (PHB 19) that of the wild type strain H16. Similarly, NADP-specific acetoacetyl-CoA reductase activities are around 50% of the wild type level.

A series of plasmids containing fragments of the *A. eutrophus* insert of plasmid pAeT29 (see Figure 1*b*; 11) were constructed in the broad host range vector pLAFR3 for complementation analysis of the PHB negative mutants (see "Materials and Methods"). Recombinant plasmids, pLA29, pLA41, and pLA42 (illustrated in Fig. 1*b*) were introduced into each of the *A. eutrophus* strains by conjugation and the resulting transconjugants analyzed on nitrogen-limited plates for the restoration of the white (PHB plus) phenotype. Plasmids pLA29, pLA41, and pLA42, each of which contains the region upstream from *phb*A-*phb*B into which Tn5 has inserted in the chromosome of strains PHB 2 and PHB 3 (Fig. 1*a*) complemented the mutation in each of these two strains, restoring the white colony phenotype. All four recombinant plasmids also restored the wild type colony phenotype to mutant strain PHB 19. In the case of this strain, the Tn5 insertion is located outside the region of the *A. eutrophus* chromosome contained in each of the plasmids (see Fig. 1*b*). The introduction of the vector pLAFR3 into each of the mutant strains failed to complement the opaque colony phenotype.

Biochemical analysis of each of the complemented strains was performed as described for the characterization of the mutants, and these results are also presented in Table II. The introduction of pLA29 (Fig. 1*b*) into each of the mutant strains, results in the restoration of PHB polymerase activity and PHB biosynthesis (Table II). In addition, an approximately 3–5-fold increase in the levels of β-ketothiolase and NADP-specific acetoacetyl-CoA reductase activities was observed. Plasmid pLA41 also restores PHB-polymerase activity and PHB production to strains PHB 2 (Table II), PHB 3, and PHB 19 (data not shown). Finally, plasmid pLA42 restores PHB polymerase activity and PHB production to all three mutant strains although the *phb*A-*phb*B genes have been deleted (Fig. 1*b*). In the case of strains containing this plasmid the β-ketothiolase and NADP-specific acetoacetyl-CoA reductase activities remain at the same level as the mutant strains (Table II). At this time we cannot make any statements with respect to the level of overproduction of PHB-polymerase in the complemented strains because of the sensitivity of the assay used. Whether or not the recombinant plasmids affect the rate or level of PHB production in *A. eutrophus* is currently under investigation.

Expression of the A. eutrophus PHB Biosynthetic Genes in E. coli—We previously noted that the *phb*A-*phb*B genes located on plasmid pAeT29 were expressed in *E. coli* under the control of the *A. eutrophus* promoter (11). Identification of the *phb*C gene upstream from *phb*A-*phb*B together with the observed decrease in thiolase and reductase enzyme activities in strains PHB 2 and PHB 3 indicates that all three genes may in fact be expressed from a single promoter located upstream from *phb*C. To study this, cultures of *E. coli* strains containing plasmids pAeT41 and pAeT42 were grown under nitrogen limiting conditions until cells reached stationary phase at which point the cells were harvested, lysed, and analyzed. *E. coli* containing pUC18 was used as a control. The results of β-ketothiolase, acetoacetyl-CoA reductase, PHB polymerase, and PHB concentration assays are presented in Table III. Only the lysate of *E. coli* containing plasmid

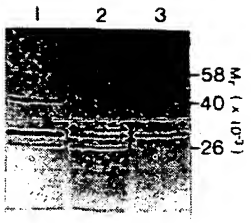

FIG. 2. **Maxicell analysis.** Maxicell labeling was performed as described under "Materials and Methods." Lanes contained lysates of *E. coli* containing plasmids pAeT10 (*lane 1*), pAeT41 (*lane 2*), and pAeT42 (*lane 3*).

pAeT41 has a significant level of each enzyme activity and PHB production, the lysate of *E. coli* containing plasmid pAeT42 which should encode a functional PHB polymerase has only 1% of the activity for this enzyme exhibited by a lysate of *E. coli* HBFR1/pAeT41.

Maxicell analysis of the *E. coli* strains described above was used to determine the molecular weight of the polypeptides encoded by plasmids pAeT41 and PAeT42. We also included plasmid pAeT10 in this analysis as this plasmid expresses the *A. eutrophus phb*A-*phb*B genes from the pUC 8 vector *lac*Z promoter (11). A typical result is shown in Fig. 2. Additional protein bands are present of M_r 40,000 and 26,000 in lanes 1 and 2 containing plasmid pAeT10 and pAeT41, respectively. Both of these plasmids express the *phb*A-*phb*B genes encoding β-ketothiolase (M_r 41,000) and NADP-specific acetoacetyl-CoA reductase (M_r 26,000; 11). Neither of these two proteins is present in the extract of cells containing plasmid pAeT42 (Fig. 2, *lane 3*) which does not contain the *phb*A-*phb*B genes. Control experiments in which the vector pUC8 was used gave no signal at M_r 41,000 or 26,000 (data not shown). Both plasmids, pAeT41 and pAeT42, express the PHB polymerase (*phb*C) gene in *E. coli* and in lanes 2 and 3 (Fig. 2) which contain extracts of cells containing these plasmids a signal at M_r 58,000 is clearly evident. Again, this protein is absent from lane 1 which contains the extract from cells containing plasmid pAeT10 which does not contain the *phb*C gene and also from a control sample of pUC8 containing cells (data not

FIG. 3. **Nucleotide sequence of the PHB polymerase (*phb*C) locus.**

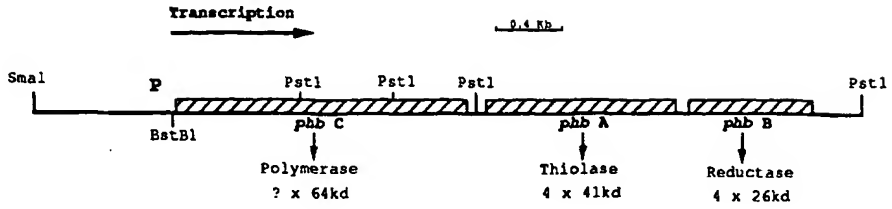

FIG. 4. **Structure of the *phb*C-*phb*A-*phb*B locus in *A. eutrophus* H16.** The insert of plasmid pAeT41 containing the *phb*C-*phb*A-*phb*B structural genes (*boxed regions*) is illustrated. The approximate location of the *phb*C promoter is shown and the gene products indicated.

shown). An additional band of around M_r 30,000 present in all three lanes in Fig. 2 was also found in control experiments of cell extracts containing pUC8 and is presumably a vector-encoded protein. From these data we conclude that the *phb*C gene expressed in *E. coli* encodes a polypeptide of approximate M_r 58,000.

Nucleotide Sequence Analysis of phbC—The 2-kb *Sma*I-*Pst*I *A. eutrophus* chromosomal DNA fragment cloned in plasmid pAeT42 contains the entire structural gene for *phb*C and probably the regulatory sequences. The sequence of this fragment was determined from both DNA strands and multiple sequence analysis by the dideoxy sequencing method as described under "Materials and Methods." A single long open reading frame extends from nucleotide 820 to a TGA stop codon at nucleotide 2608. Potential translation initiation codons are present at position 842 (ATG), 1067 (ATG), and 1097 (ATG). Translation from each of these potential start sites would produce proteins of M_r 63,940, 55,513, and 54,483, respectively. As there is no NH_2-terminal sequence data available for PHB polymerase, the unequivocable identification of the correct translation start site is not possible at this time. However, significant amino acid sequence homology between the translation product from the ATG at position 842 to the ATG at position 1067 and the *P. oleovarans* PHA polymerase gene product[3] indicates that the first ATG (position 842) is probably correct. We are currently purifying a *phb*C-*lac*Z fusion protein for NH_2-terminal sequence analysis. Fig. 3 presents the entire nucleotide sequence of this region from the *Sma*I site to the first 30 nucleotides of the *phb*A gene (11) located downstream. The translation product of the open reading frame from the ATG at position 842 to the TGA at position 2609 is also shown. Hence PHB polymerase encoded by the *phb*C gene in plasmid pAeT42 is a polypeptide of 589 amino acids with an $M_r = 63,940$. The NH_2-terminal 10 amino acids of the *phb*A gene product are also presented in Fig. 3 (11). Additional features of the nucleotide sequence presented in Fig. 3 include the COOH-terminus of an open reading frame that begins upstream from the *Sma*I site and terminates at the TGA stop codon at position 76. Identification of the *phb*C promoter sequences requires additional experiments. Located 85 bp downstream from the *phb*C TGA stop codon (position 2609) lies the ATG start codon for the *phb*A structural gene (position 2969). The function of the hairpin structure in this region (indicated on Fig. 3) remains unclear at this time. From these data it is clear that the three enzymes of the *A. eutrophus* PHB biosynthetic pathway are encoded by three genes organized as *phb*C-*phb*A-*phb*B as illustrated in Fig. 4.

DISCUSSION

The location of the gene encoding PHB polymerase was identified by analyzing and complementing a series of PHB negative mutants of *A. eutrophus* H16. Two Tn5 insertion mutations (PHB 2 and PHB 3) which eliminate the synthesis of PHB (Table II) were mapped by DNA hybridization 1.2 kb (PHB 2) and 1.5 kb (PHB 3) upstream from the *phb*A-*phb*B structural gene in plasmid pAeT29 (Fig. 1*a*). A third type of mutant, strain PHB 19, had a Tn5 insertion located outside the region of the *A. eutrophus* chromosome cloned in plasmid pAeT29 yet had a similar phenotype to strain PHB 2 and strain PHB 3 (Table II). All three of these mutant strains were complemented by a restriction fragment extending from the first *Sma*I site 2.2 kb upstream of *phb*A to the *Pst*I site located 40 bp upstream from *phb*A, plasmid pLA42 (Fig. 1*b*). Nucleotide sequence analysis of this region identified the PHB polymerase gene (*phb*C) immediately upstream from *phb*A as illustrated in Fig. 4. From the sequence it is clear that for both strain PHB 2 and strain PHB 3, Tn5 has inserted within the structural gene for PHB polymerase. These mutations eliminate PHB polymerase activity and reduce by around 55% the activities of β-ketothiolase and NADP-specific acetoacetyl-CoA reductase (Table III). As *A. eutrophus* contains at least two β-ketothiolase and acetoacetyl-CoA reductase enzymes (25, 26), it is unclear at this stage if the residual activities observed in these strains are due to reduced expression of *phb*A-*phb*B, or the other enzymes. It is possible that the additional thiolase and reductase enzymes acting with enoyl-CoA hydratase and/or an epimerase can synthesize D(−)-3-hydroxybutyryl-CoA. This would then explain why we did not find any Tn5 insertions in the *phb*A-*phb*B structural genes, *i.e.* mutant strains carrying Tn5 insertions in either of these two genes would still be capable of synthesizing the substrate for PHB-polymerase and therefore produce PHB. The organization of the *phb*C-*phb*A-*phb*B genes (Fig. 4) suggests that they are all expressed from the *phb*C promoter. At this stage we cannot rule out a second promoter upstream from *phb*A-*phb*B which could account for some of the residual thiolase and reductase activities observed for strain PHB 2 and strain PHB 3. We are now examining a series of *lac*Z fusions to determine if this is the case. The nature of the mutation in strain PHB 19 which significantly reduces PHB production remains unclear at this time as there is no Tn5 insertion near the *phb*C-*phb*A-*phb*B genes in this strain. It is possible that the Tn5 insertion affects a regulatory gene, but we consider it more likely that it is a spontaneous mutation in the *phb*C promoter.

The presence of the *A. eutrophus phb*C-*phb*A-*phb*B genes in *E. coli* results in the accumulation of large quantities (around 50% dry cell weight) of PHB when the recombinant cells are grown under nitrogen limitation (Table III). This result was recently confirmed by two other groups of investigators (27, 28), although the gene organization was not reported in either case. Interestingly, the expression of *phb*C alone in *E. coli* produces neither PHB or significant levels of PHB polymerase activity (plasmid pAeT42, Table III). *E. coli* appears incapable of synthesizing D(−)-3-hydroxybutyryl-CoA, as substrate for PHB polymerase, in the absence of the *A. eutrophus phb*A-*phb*B genes. As we know the insert of pAeT42 contains both the promoter and structural gene for *phb*C (plasmid pLA42 complements all PHB negative mutants, Table II), we postulate that in the absence of available substrate, PHB polymerase may be inactive or rapidly de-

[3] O. P. Peoples, manuscript in preparation.

graded in *E. coli*. It is possible that an interaction of the polymerase with the thiolase and/or reductase proteins is required for functional polymerase activity. However, we consider this unlikely as granule-bound PHB polymerase is both active and stable (Table III), yet all of the thiolase and reductase enzyme activities remain in the soluble fraction of the cell extracts.

The nucleotide sequence of the *A. eutrophus* chromosomal DNA insert in plasmid pLA42 encoding PHB polymerase predicts a single polypeptide of M_r 63,940 (Fig. 3). Although PHB polymerase has not previously been purified and its subunit molecular weight determined, the results of *E. coli* maxicell studies (Fig. 2) indicate an M_r = 58,000 for this polypeptide, in reasonable agreement with that predicted from the gene sequence. For a number of years, it was proposed that the polymerization of D(−)-3-hydroxybutyryl-CoA involves a membrane-bound polymerase which forms a type of barrier between the aqueous environment of the cytoplasm and the hydrophobic crystalline PHB granules (29). The hydropathy profile of the PHB polymerase polypeptide does not indicate a typical membrane spanning structure. In addition, NMR studies of native PHB granules in *Methylobacterium* (30) indicates that these granules are in a mobile as opposed to a highly crystalline solid state. Together these data lend credence to the idea that PHB biosynthesis does not in fact require a complex membrane-bound polymerization system. The mechanism for PHB polymerase in the literature proposed by Griebel and Merrick (31) involves two partial reactions. Acyl-*S*-enzyme intermediate formation in the first reaction is followed by transfer to a primer acceptor in the second reaction. The predicted primary structure of PHB polymerase (Fig. 3) has 5 cysteine residues, Cys^{246}, Cys^{319}, Cys^{382}, Cys^{438}, and Cys^{459}. It is worth noting at this stage that only two of these residues, Cys^{319} and Cys^{459}, are conserved in the *P. oleovarans* PHA polymerase sequence.[3]

The characterization of *phb*C enables us to initiate attempts to overproduce the gene product in *E. coli* for mechanistic and substrate specificity analysis. In view of the range of substrates which the enzyme has so far been shown to use (2–5), perhaps PHA polymerase would be a more correct name. The mechanism(s) by which PHB is accumulated on depletion of a specific nutrient in the growth medium remains unclear. However, reports that β-ketothiolase and NADP-specific acetoacetyl-CoA reductase are constitutive (25, 26) together with the organization of the genes in *A. eutrophus* (Fig. 4) and the requirement of a functional *phb*C for optimum levels of expression of *phb*A-*phb*B (Table II) may indicate that all three enzymes are constitutively expressed. In this scenario, the accumulation of PHB would be dictated entirely by the intracellular levels of the substrates and cofactors and the kinetic parameters of the three enzymes. What role the other thiolase and reductase enzyme activities, detected in *A. eutrophus* (25, 26), play in determining the intracellular level of D(−)-3-hydroxybutyryl-CoA remains unclear at this time. The observed lability of the *phb*C gene product in *E. coli* in the absence of *phb*A-*phb*B expression (plasmid pAeT42, Table III) together with the reported instability of PHB polymerase in *Z. ramigera* cells not producing PHB (8) are consistent with the hypothesis that in the absence of substrate PHB polymerase is unstable and degraded. Experiments to test this hypothesis and elucidate the mechanisms by which PHB metabolism is regulated are currently underway.

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Gene, 167 (1995) 197–201

GENE 09347

Isolation and characterization of a cDNA encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl-coenzyme A synthase

(*bap1* yeast mutant; isoprenoid; *Saccharomyces cerevisiae*; sterol)

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SUMMARY

An 1.7-kb *Arabidopsis thaliana* (*At*) cDNA was isolated by complementation of a *bap1* mutation affecting the transport of branched-chain amino acids (aa) in the yeast *Saccharomyces cerevisiae*. The determination of the nucleotide (nt) sequence revealed an open reading frame of 1383 nt which may encode a protein of 461 aa with a predicted molecular mass of 51 038 Da. The deduced aa sequence exhibited strong similarities with mammalian 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS) sequences. Although former biochemical studies have suggested that acetoacetyl-coenzyme A thiolase (AACT) and HMGS activities were carried by a single protein in plants, complementation studies and measurements of enzymatic activities clearly showed that the *At* HMGS is devoid of AACT activity.

INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) plays a key role in the synthesis of various sterols and isoprenoids in eukaryotic cells. Sterols are structural components of plasma membranes, whereas terpenoids are involved in various functions including respiration, glycosylation and signal transduction (Goldstein and Brown, 1990). The isoprenoid pathway of plants is characterized by its diversity, which leads to the synthesis of various molecules such as phytohormones (gibberellins and abscisic acid) and secondary metabolites (aromatic terpenoids and phytoalexins). HMG-CoA is also a putative intermediate in the degradation of branched-chain amino acids (aa).

In yeast and mammalian tissues, the conversion of three acetyl-CoA molecules to one molecule of HMG-CoA is mediated by two distinct enzymes, i.e., acetoacetyl-coenzyme A thiolase (AACT; EC 2.1.3.9), and HMG-CoA synthase (HMGS; EC 4.1.3.5). In plants, this conversion has been described as mediated by a membraneassociated enzyme which seems able to catalyze both reactions, and is therefore referred to as an AACT/HMGS enzyme system (Weber et Bach, 1994). SDS-PAGE of the purified enzyme system showed a single protein band at 55.5 kDa (Weber and Bach, 1994), and AACT and HMGS activities comigrate after various chromatographical separation procedures (Van der Heijden et al., 1994). These results, and detailed analysis

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Abbreviations: aa, amino acid(s); AACT, acetoacetyl-coenzyme A thiolase; *At*, *Arabidopsis thaliana*; *bap1*, mutant gene encoding branched-chain aa permease; bp, base pair(s); *erg10*, mutant gene encoding AACT; *erg11* and *erg13*, mutant genes encoding HMGS; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGS, HMG-CoA synthase; kb, kilobase(s) or 1000 bp; mt, mitochondrial; nt, nucleotide(s); ORF, open reading frame; *PGK*, gene encoding phosphoglycerate kinase; *PMA*, gene encoding the plasma membrane ATPase; Smm, sulfonylmeturon methyl; *TRP1*, gene encoding *N*-(5'-phosphoribosyl) anthranilate isomerase; *URA3*, gene encoding orotidine-5'-monophosphate decarboxylase; *UTR*, untranslated region(s); wt, wild type; [], denotes plasmid-carrier state.

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of the activities detected after feeding of ^{14}C-labeled acetyl-CoA (Bach et al., 1990), leave open the possibility that both activities would be carried by only one protein, thus channeling a directed flux of carbon units into the isoprenoid pathway (Bach et al., 1994).

EXPERIMENTAL AND DISCUSSION

(a) Isolation of an *At* cDNA encoding HMGS

HMGS cDNA was cloned in the course of attempts to isolate a cDNA encoding a plasma membrane permease mediating the transport of branched-chain aa. The yeast strain C3023 (*MATα bap1 ura3*-1 *trp1*), deficient in the transport of branched-chain aa (Tullin et al., 1991) was complemented by an *At* cDNA library constructed in the yeast expression vector pFL61 (Minet et al., 1992). Complemented yeasts were screened on a medium containing branched-chain aa and sulfonylmeturon methyl (Smm) (Dupont de Nemours). Smm is an inhibitor of acetolactate synthase (EC 4.1.3.18) the enzyme which controls the biosynthesis of these aa. The rationale of the screening was that, even in the presence of branched-chain aa, the *bap1* yeast mutant would not grow in the presence of Smm; in contrast, complementation by a cDNA encoding a permease transporting these aa was expected to restore growth.

Yeast was transformed by electroporation (Becker and Guarente, 1991), and Ura$^+$ transformants were first selected on minimal medium supplemented with 50 μg Trp/ml. The Ura$^+$ transformants (8600) were then replicated on the same medium supplemented with Leu, Ile and Val (50 μg/ml each) and Smm (20 μg/ml). One of the transformants showed a partially restored uptake of Val and Leu (data not shown). Plasmid pFLAE was isolated from this clone and a restriction map is presented in Fig. 1A.

(b) cDNA sequence analysis

Both strands of the 1.7-kb insert contained in pFLAE were sequenced using the dideoxy chain-termination method (Sanger et al., 1977). The full-length spans 1625 nt and contains a 1383-bp ORF (see Fig. 1B), assuming that the first ATG codon at nt 59 is the translational start. The sequence surrounding this ATG codon fits with the plant consensus sequence AAC*AATGGC (Lütcke et al., 1987). The 3′-*UTR* (181 bp) contains a 6-bp sequence (AACAAA) described as a putative polyadenylation signal (Joshi, 1987) at nt 1457. Two other putative consensus motifs have been found in this region. One is located 24 bp downstream from the polyadenylation signal and may be involved in the efficiency of polyadenylation; the other one flanks the polyadenylation site and is supposed to control the precise selection of cleavage site (Joshi, 1987).

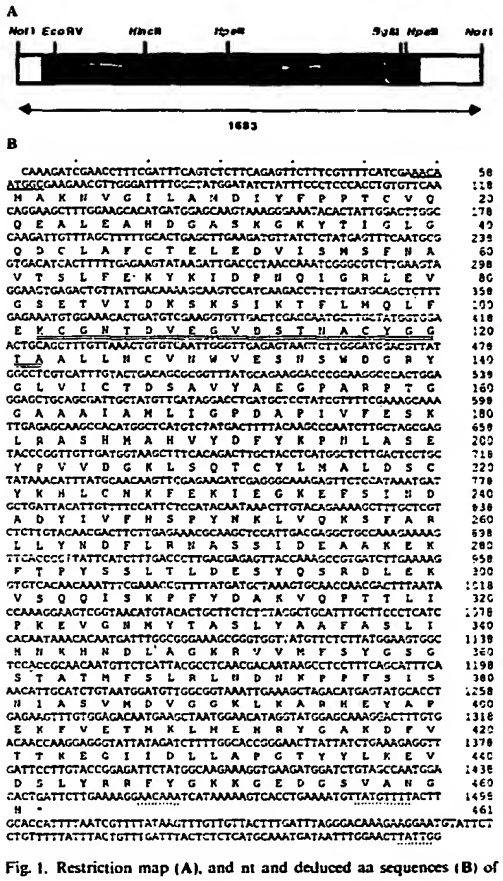

Fig. 1. Restriction map (A), and nt and deduced aa sequences (B) of *At HMGS* cDNA. (A) The 1683-bp *Not*I-*Not*I fragment corresponds to the entire cDNA. The *Not*I sites are originated from the vector. The coding region is represented by a solid box. (B) This fragment was subcloned in pBluescript KS$^+$ and both strands were sequenced with T7 polymerase from a set of overlapping exonuclease-III-generated deletions and using synthetic oligodeoxynucleotides. The nt sequence data reported appear in the EMBL database under accession No. X83882. The plant consensus initiation sequence surrounding the start codon is underlined. The regions underlined by the bold dashed lines correspond to the putative polyadenylation signals as described in section b and the putative active site is double underlined.

(c) Analysis of the deduced aa sequence

The deduced plant HMGS sequence (461 aa, 51038 Da) is smaller than the corresponding enzymes of chicken liver (522 aa, 57494 Da), rat liver (520 aa, 57369 Da), human fibroblastic tissue (520 aa, 57287 Da) and chinese hamster ovary cells (520 aa, 57254 Da). The plant

sequence revealed 43, 42, 42 and 41% identity with the mammalian cytoplasmic enzymes, respectively, and was also 42% identical to the mt HMGS sequence from rat (508 aa, 56847 Da). However, the plant sequence does not contain a sequence analogous to the 37-aa signal sequence found at the N-terminal end of the rat mt enzyme.

At and mammalian HMGS are highly homologous in aa residues, mainly within the N-terminal domain where the catalytic site is located. Miziorko and Behnke (1985) have sequenced the putative active site region of the mt HMGS from chicken liver. Comparison of this sequence with the corresponding region of the *At* HMGS showed that the putative active site is strongly conserved in animal and plant species. Likewise, the Cys^{117} found in the plant sequence may correspond to the Cys which has been suggested to be involved in the formation of the acyl-*S*-enzyme intermediate (Vollmer et al., 1988). The plant deduced HMGS protein shows a potential *N*-linked glycosylation site at aa 269.

(d) Southern blot analysis

A Southern blot analysis under high stringency was made to check whether the isolated cDNA really derived from the *At* genome. Genomic DNA extracted from *At* seedlings was cut with several restriction enzymes and the fragments were probed with a 1.25-kb *Eco*RV-*Bgl*II fragment from the *At* cDNA (Fig. 1A). No hybridizing band was detected for the yeast genomic DNA used as a control (data not shown). Digestions with *Eco*RV + *Bgl*II and *Eco*RV + *Bgl*II, which all cleave the DNA outside the sequence probed yielded a strong hybridization signal (Fig. 2, lanes a, b, c). Digestions with *Hpa*II (Fig. 2, lane e), which cuts between the *Eco*RV and *Bgl*II sites (Fig. 1A) yielded the two expected major bands. However, digestion with *Hin*cII, which cuts in the same region, yielded three bands (Fig. 2, lane d). The *HMGS* sequence, therefore, clearly belonged to the *At* genome. The additional band obtained after *Hin*cII digestion may result from the presence of an intron inside HMGS genomic DNA. Whatever the enzymes used, minor hybridization signals could be detected. This suggests that another gene related to the *HMGS* gene could be present in the plant genome.

(e) Expression of *HMGS* in yeast

Sterol auxotrophic mutant strains defective in HMGS activity belong to two complementation groups (*erg11* and *erg13*; Servouse et al., 1984). To check whether the plant gene was able to complement *erg11*-1 and *erg13*-1 mutations, mutant strains were transformed with pFLAE. The transformants were not clearly relieved for sterol auxotrophy. In vitro enzymatic measurements showed

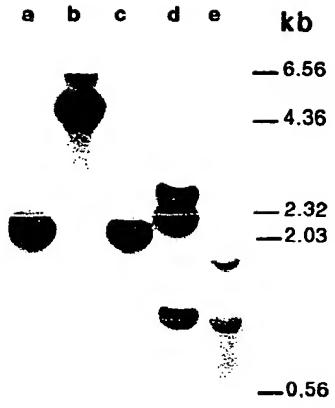

Fig. 2. Southern blot analysis of genomic *At* DNA. Lanes a–e: 2 μg per lane of *At* genomic DNA digested with *Eco*RV + *Bgl*II, *Eco*RV, *Bgl*II, *Hin*cII and *Hpa*II, respectively. The molecular size standards are shown to the right. Methods: Genomic DNA was isolated from *At* seedlings (Rogers and Bendich, 1985) and digested with several restriction endonucleases. The fragments were electrophoretically fractionated and transferred to a nylon membrane (Southern, 1975). The 1.25-kb *Eco*RV-*Bgl*II fragment (Fig. 1A) was purified from agarose gel. ^{32}P-labeled by random priming and used as a probe. Hybridization and filter wash were performed at high stringency (0.015 M NaCl/0.0015 M Na_3-citrate pH 7.0) at 65°C as described by Sambrook et al. (1989).

that the bad complementation was related to absence of noticeable HMGS activity in transformed cells.

Therefore, cDNA was subcloned into the NEV expression vector containing the yeast *PMA1* promoter instead of the *PGK* promoter of pFL61. The *PMA1* promoter has been shown to give high expression of plant cDNAs in yeasts (Sauer and Stolz, 1994). The expression product of pNEVAE complements both *erg11*-1 and *erg13*-1 mutations (Fig. 3). Fig. 3 also shows that pNEVAE did not complement the *erg10*-1 mutation carried by F2SP5 (*erg10*) mutant strain which is defective in AACT activity (Dequin et al., 1988).

HMGS and AACT activities of wt FL100 strain, *erg11* mutant and transformed strain *erg11*-AE were measured on cell-free extracts as described earlier (Servouse and Karst, 1986). Specific activity of HMGS was increased 15-fold in *erg11* yeast mutant carrying pNEVAE in comparison with the wt strain (Table I). AACT activity was similar in the transformed and non-transformed mutant strains (Table I).

(f) Conclusions

(*1*) These data characterize for the first time a plant *HMGS* cDNA. The deduced plant enzyme exhibits strong homologies with the corresponding animal enzymes, particularly in the region of the active site, located between aa 102 and 122.

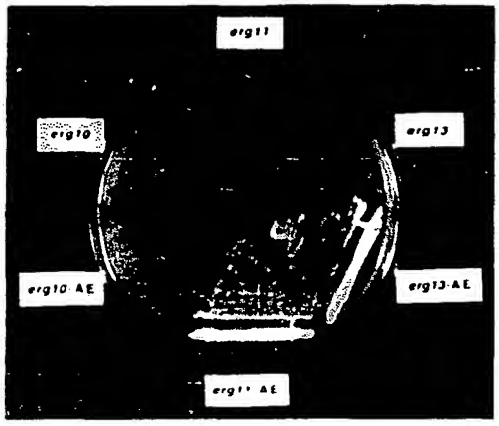

Fig. 3. Complementation assays of temperature-sensitive mutant strains defective in HMGS (*erg11* and *erg13*) and AACT (*erg10*) activities with plasmid pNEVAE. Growth of transformant clones (*erg10*-AE, *erg11*-AE and *erg13*-AE) and of the corresponding mutants strains on complete medium, after 72 h incubation at 36°C.

TABLE I

HMGS and AACT activities of FL100, *erg11* and *erg11*-AE

Strain[a]	HMGS activity[b]	AACT activity[b]
FL100	17.3	140
erg11	ND	191
erg11-AE	230	219

[a]Yeast strains: FL100 (ATCC 28383, *Mat* a) is the parent strain. *erg11* (*erg11*-1, *ura3*-1) is derived from FL100 and *erg11*-AE is the transformed strain of genotype (*erg11*-1, *ura3*-1 [pNEVAE]).
[b]Activities are expressed as nmol acetoacetyl-CoA/min per mg protein. Data are representative of twice repeated experiments. ND, not detected.

(2) The complementation and enzymatic data demonstrate that the peptide encoded by the *At* cDNA is devoid of AACT activity. Therefore, it is likely that in plants the synthesis of HMGS takes place through two subsequent steps involving independent AACT and HMGS activities, as described in animal and yeast cells. These data seem to contradict the biochemical data according to which radish AACT and HMGS activities are carried by a single monomeric protein (Weber and Bach, 1994).

(3) It is not yet completely clear why the screening assay based on Smm resistance led to the isolation of the *HMGS* clone. One first hypothesis is that *bap1* strain was defective in HMGS activity and that the consecutive low sterol amount of the strain could be at the basis of impairment of branched-chain aa uptake. However, this possibility was excluded by sterol analysis and measurements of enzymatic activity (data not shown). Nonetheless, HMGS activity is likely related to aa uptake, because complementation of the *bap1* mutant by plant HMGS strongly stimulated the uptake of Leu, Val and Glu (data not shown). The nature of the relationship between HMGS activity and aa uptake remains to be investigated.

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ORIGINAL PAPER

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Cloning and characterization of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase in melon (*Cucumis melo* L. *reticulatus*)

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Abstract We have isolated a cDNA for *Cm-HMGR*, encoding 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in melon (*Cucumis melo* L. *reticulatus*; Genbank Accession No. AB021862). *Cm-HMGR* encodes a polypeptide of 588 amino acids that contains two transmembrane domains and a catalytic domain. Database searches revealed that Cm-HMGR shows homology to HMG1 (63.7%) and HMG2 (70.3%) of tomato, to HMG1 (77.2%) and HMG2 (69.4%) of *Arabidopsis thaliana*, and to *HMGR* of tobacco (72.6%). Functional expression in a HMG-CoA reductase-deficient mutant yeast showed that *Cm-HMGR* products mediate the synthesis of mevalonate. Northern analysis revealed that the level of *Cm-HMGR* mRNA in the fruit increased after pollination and markedly decreased at the end of fruit enlargement. During ripening, *Cm-HMGR* mRNA levels increased markedly in the fruit. In parallel with mRNA expression, Cm-HMGR activity increased after pollination, whereas no Cm-HMGR activity was detectable during fruit ripening. Our results suggest that *Cm-HMGR* is important during early post-pollination development of the fruit in melon.

Key words Melon · 3-Hydroxy-3-methylglutaryl coenzyme A reductase gene · Fruit development · Yeast complementation

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Introduction

A leading issue in the development of higher plants is the determination of fruit size. To elucidate the mechanism responsible, Higashi and colleagues (1999) observed the histology of cells during fruit development in two melon genotypes whose mature fruits differed in size. The results suggested that the degree of cell proliferation during early fruit development determines fruit size in melon and that temperature affects the factor that regulates the extent of cell proliferation. However, the precise mechanism by which cell proliferation is regulated remained unclear.

Using tomato, Narita and Gruissem (1989) demonstrated that expression and activity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) encoded by *HMG1* are required during early fruit development. Histochemical analysis of transgenic tomato strains carrying *HMG1::GUS* reporter gene fusions, as well as of the transcription of *HMG1* and the activity of HMGR in suspensions of tomato cells, suggested that a primary role of *HMG1* is to meet the demand for mevalonate that is associated with cell division and growth (Jelesko et al. 1999). In addition, Cowan et al. (1997) used normal and small-fruit phenotypes of avocado to probe the interaction between the end products of isoprenoid biosynthesis and the activity of HMGR in the metabolic control of fruit growth. The results suggested that accumulation of abscisic acid down-regulates mesocarpal HMGR activity and decreases fruit size by reducing the number of cells. These findings in tomato and avocado indicate the involvement of HMGR in fruit development.

End products of isoprenoid synthesis are very important in many biological processes in plants, such as the synthesis of membrane sterols and plant growth hormones (cytokinin, abscisic acid, gibberellins, and brassinosteroids), electron transport (cytochrome *a*, quinones, chlorophyll), isoprenylation of proteins, and production of sesquiterpenoid phytoalexins, which

provide resistance to pathogens (Chappell 1995; McGarvey and Croteau 1995; Weissenborn et al. 1995; Westwood et al. 1998). Mevalonate is essential for isoprenoid synthesis in plants, and HMGR is the key enzyme of isoprenoid synthesis, catalyzing the conversion of HMG-CoA into mevalonate.

HMGR genes already have been isolated from many plant species, such as *Arabidopsis* (Caelles at al. 1989; Learned and Fink 1989; Enjuto et al. 1994), potato (Choi et al. 1992; Bhattaharyya et al. 1995; Korth et al. 1997), tomato (Park et al. 1992), *Nicotiana sylvestris* (Genchik et al. 1992), para rubber tree (*Hevea brasilensis*; Chye et al. 1991, 1992), *Catharauthus roseus* (Maldonado-Mendoza et al. 1992), wheat (Aoyagi et al. 1993), cotton (Loguercio et al. 1999), and mulberry (Jain et al. 2000). The structure of plant HMGRs differs markedly from that in animals and fungi. The HMGRs found in animal and fungi have seven membrane-spanning domains in the N-terminal domain (Basson et al. 1988), whereas HMGRs isolated from higher plants usually have only two membrane-spanning domains (Learned and Fink 1989). Regardless of the number of membrane-spanning regions, HMGR is membrane bound (Chye et al. 1992).

In the present study, we isolated and characterized a gene encoding HMGR from melon and analyzed its transcription during fruit development. In addition, we discuss the possible role of melon HMGR in fruit growth.

Materials and methods

Plant material and growth

We used two cultivars of melon (*Cucumis melo* L. *reticulatus* cv. Fuyu A and Natsu 4) and their hybrid, Fuyu A × Natsu 4. For surface sterilization, melon seeds were soaked in 70% (v/v) ethanol for 15 s then in 1% (v/v) hypochlorite solution for 15 min; treated seeds were rinsed three times with distilled water. For RT-PCR, surface sterilized Fuyu A seeds were germinated on Murashige and Skoog (1962) medium (pH 5.8) with 3% (w/v) sucrose and 0.4% (w/v) gellan gum (Gelrite, Wako Pure Chemicals, Osaka, Japan) and grown in darkness at 25 °C for 10 days. The seedlings were harvested, then frozen in liquid nitrogen and stored at −80 °C until use. For other applications, seeds of the three genotypes were sown and grown in greenhouses. Freshly opened female flowers were hand-pollinated, and one fruit per plant was allowed to develop. Fruit was harvested at various times after pollination, and the pericarp tissue was separated from the contents of the seed cavity, sliced, frozen in liquid nitrogen, and stored at −80 °C until use.

Cloning of a *HMGR* homolog from a melon cDNA library

During a screen for *ETR* homologs in melon, we serendipitously isolated a clone encoding a protein that was highly homologous to other plant HMGRs (Sato-Nara et al. 1999) from a cDNA library generated from RNA isolated from melon fruits 50 days post-pollination. The cDNA fragment was subcloned in pBSII SK+ (Clontech, Palo Alto, Calif., USA). The inserts were sequenced with a 373S DNA sequencing system and ABI PRISM Dye Primer Cycle Sequencing-Ready Reaction Kits (Perkin-Elmer Cetus, Norwalk, Conn., USA). We used Genetix-Mac version 7.3 (Software Development Co., Tokyo, Japan) to analyze sequences.

Yeast growth and transformation

We used the *Saccharomyces cerevisiae* strain JRY2394 (*MAT*a, *ade2*, *his3*, *met-*, *ura3*, *hmg1*, *hmg2*; kindly provided by Dr. Jasper Rine, U.C. Berkeley, Calif., USA) to investigate the function of *Cm-HMGR*. The yeast strain has loss-of-function mutations in two HMGR genes, *HMG1* and *HMG2*, and requires mevalonate for growth. The wild-type yeast strain TM 100 (*ura3*, *leu2*, *trp1*) was used as a control.

We cloned the *Cm-HMGR* cDNA into the pYES2 expression vector (Invitrogen) and transformed JRY2394 as described by Kaiser et al. (1994). The empty pYES2 vector was used as a control. *Cm-HMGR* transformants were selected on YM galactose medium containing 0.67% yeast nitrogen base without amino acids, 2% galactose, and 30 mg/l each of adenine, histidine, and methionine. For non-selective conditions, we added 5 mg/l mevalonate and 2% glucose instead of galactose.

Genomic Southern analysis

We isolated genomic DNA from young leaves of Fuyu A, Natsu 4, and the Fuyu A × Natsu 4 hybrid according to Wagner et al. (1987). Digested DNA (20-µg aliquots) was electrophoresed in a 0.8% agarose gel and transferred to Hybond-N+ (Amersham-Pharmacia Biotech, Tokyo, Japan). Using a 1.25-kb *Sal*I-*Bgl*II fragment encoding the catalytic domain as a probe, hybridization was performed as described by Church and Gilbert (1984) at 60 °C (low stringency) or 65 °C (high stringency) for 16 h. Results were visualized by autoradiography at −80 °C.

Northern analysis

We extracted total RNA from melon fruit at various developmental stages according to the protocol of Sato-Nara et al. (1999). We followed the method of Collart and Oliviero (1993) to isolate total RNA from yeast. We fractionated 10 µg of the total RNA on a 1.0% agarose/formaldehyde gel (Berk and Sharp 1978), and transferred the RNA to Hybond-N+ (Amersham). Using the 1.25-kb *Sal*I-*Bgl*II fragment encoding the catalytic domain as a probe, Northern hybridization was performed overnight at 65 °C in a solution containing 10% sodium dextran sulfate, 1% SDS, 1 M sodium chloride, 100 µg/ml denatured salmon sperm DNA, and the ^{32}P-labeled probe. After hybridization, the membranes were rinsed twice for 15 min each in 2 × SSC at room temperature and once with 2 × SSC containing 1% SDS for 30 min at 65 °C. The filters then were autoradiographed at −80 °C.

HMG-CoA reductase assay

To isolate microsomal membranes, pericarp was pulverized in liquid nitrogen, and 10 g of the powdered pericarp was homogenized in 40 ml of extraction buffer (10 mM TRIS-HCl pH 7.0, 0.35 M sucrose, 30 mM EDTA pH 8.0, and 10 mM β-mercaptoethanol). The homogenate was filtered through two layers of cheesecloth and centrifuged at 12,000 ×*g* for 10 min; the resulting supernatant was centrifuged at 50,000*g* for 60 min. The pellet was resuspended in a medium containing 0.2 M potassium phosphate (pH 6.9) and 25 mM DTT and the resulting suspension was used to assay HMGR activity. Yeast protein extract was prepared according to the method of Basson et al. (1987); protein concentration was determined by using the Protein Assay kit (Bio-Rad, Hercules, Calif., USA).

We used the radiometric assay of Oba et al. (1985), with slight modifications, to measure HMGR activity. The reaction mixture contained 1 µl of 0.2 M DTT, 2 µl of an NADPH-generating system (20 mM NADP, 100 mM glucose 6-phosphate and 0.01 U/µl glucose 6-phosphate dehydrogenase), 3 µl of [3-^{14}C]HMG-CoA (0.34 nM/µl, 740 kBq/ml, Amersham) and an amount of microsomal fraction equivalent to 10 µg protein in a total volume of 20 µl. The reaction was started by adding [3-^{14}C]HMG-CoA, and was stopped after 20 min incubation at 30 °C by adding 2 µl of 1 M MVA and 2 µl of 6 M HCl. Further incubation at 25 °C for 15 min

to ensure lactonization. After centrifugation, 10 µl of the supernatant solution was applied to a TLC plate (25 TLC aluminium sheets 20 × 20 cm, Silica gel 60, Merck), and developed with chloroform-acetone (2:1 v/v). The plate was exposed to iodine vapor to visualize mevalonolactone. Radioactivity was measured using a Storm860 Fluorescence Imager with Image Quant software (Molecular Dynamics, Sunnyvale, Calif., USA). One unit of HMG-CoA reductase activity was defined as the amount of enzyme that formed 1 nmol of mevalonate in 1 h at 30 °C.

Results

Molecular cloning of *Cm-HMGR*

We isolated a 2357-bp cDNA clone corresponding to the *HMGR* gene from a cDNA library prepared with RNA isolated from mature melon fruits. The deduced amino acid sequence (588 residues) showed homology to tomato HMG1 (63.7%) and HMG2 (70.3%), to *Arabidopsis* HMG1 (77.2%) and *Arabidopsis* HMG2 (69.4%), and was 72.6% homologous to tobacco HMGR (Fig. 1). Like other HMGRs from plants, the deduced protein has two predicted membrane-spanning domains, which are localized in the N-terminal region, and b1 and b2 domains, which represent the putative catalytic domains. Therefore we designated the corresponding gene *Cm-HMGR* (for *C. melo* 3-*h*ydroxy-3-*m*ethylglutaryl CoA *r*eductase; Genbank Accession No. AB021862).

Phylogenic analysis showed that plant HMGRs separated into two or three groups, and HMGRs from the same or related plant species tend to belong to the same

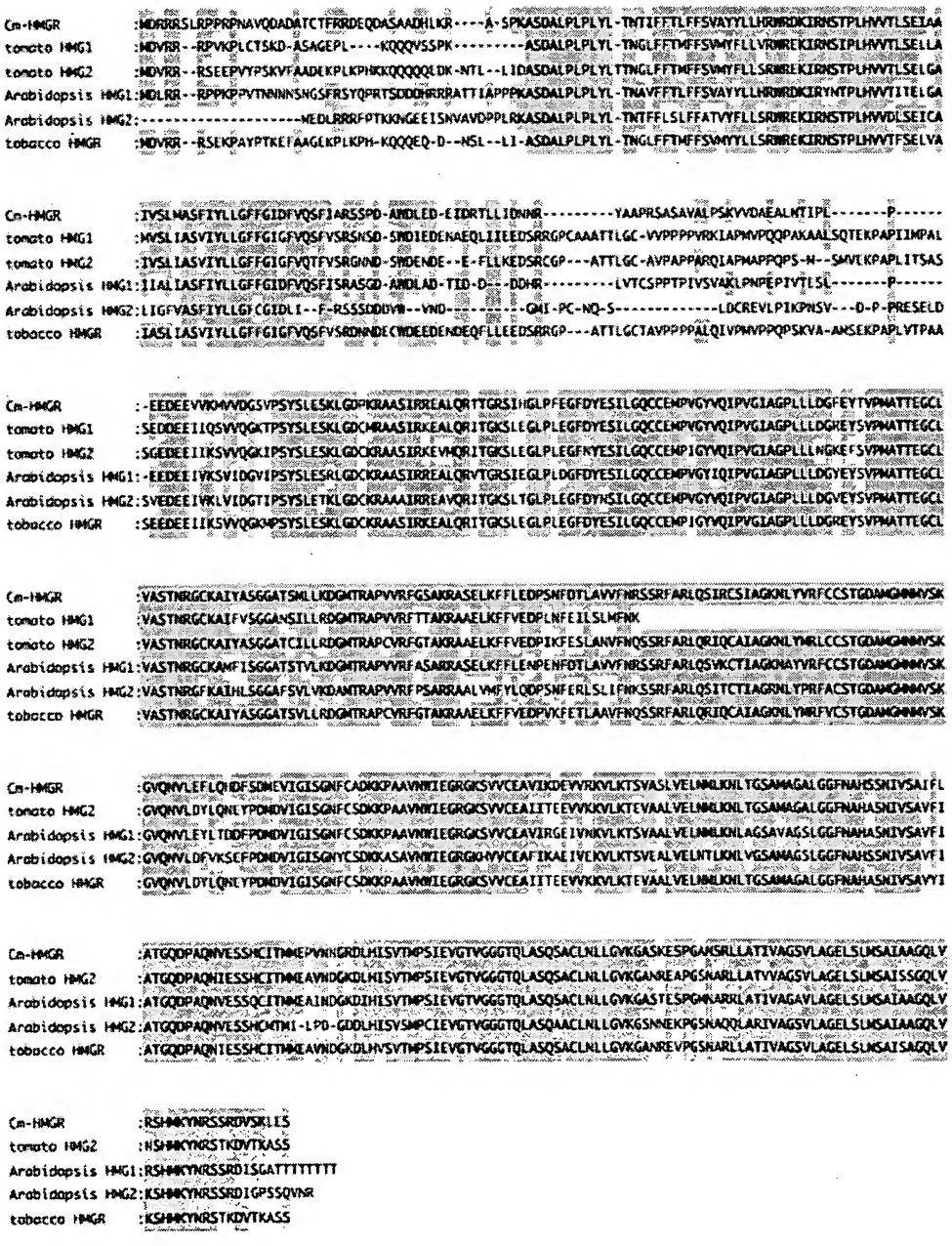

Fig. 1 Comparison of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase from melon (Cm-HMGR), tomato, *Arabidopsis* and tobacco. The alignment was performed using GENETYX version 10.1 (SDC Software, Tokyo Japan)

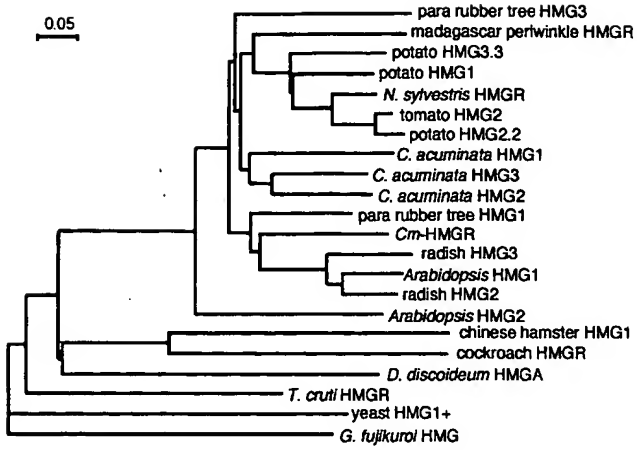

Fig. 2 Relationships between the primary amino acid sequences of HMGRs from various plants, animals, and fungi. The phylogenetic tree was constructed by using CLUSTAL X (Thompson et al. 1997). The number next to the node represents the bootstrap value from 1000 replicates. Deduced amino acid sequences were retrieved from the Genbank, EMBL and DDBJ databases. The Accession Nos. are: para rubber tree HMG3, U72145; Madagascar periwinkle HMGR, M96068; potato HMG3.3, U51986; potato HMG1, L01400; *Nicotiana sylvestris* HMGR, X63649; tomato HMG2, M69642; potato HMG2.2, U51985; *Camptotheca acuminata* HMG1, L10390; *C. acuminata* HMG3, U72145; *C. acuminata* HMG2, U72146; para rubber tree HMG1, X54659; Cm-HMGR, AB021862; radish HMG3, X68652; *Arabidopsis* HMG1, L19261; radish HMG2, X68651; *Arabidopsis* HMG2, L19262; Chinese hamster HMG1, L00165-L00183; cockroach HMGR, X70034; *Dictyostelium discoideum* HMGA, L19349; *Trypanosoma cruzi* HMGR, L78791; yeast HMG+, L76979; and *Gibberella fujikuroi* HMG, X94307

group (Fig. 2). CM-HMGR and tomato HMG2, both of which occur in developing fruit, belonged to different groups. Unfortunately, the complete sequence of tomato HMG1, another HMGR found in developing fruit, is not yet available. Therefore, we could not ascertain whether CM-HMGR and tomato HMG1 belong to the same group.

Functional analysis of *Cm-HMGR*

To analyze the function of Cm-HMGR, the cDNA was expressed in the yeast strain JRY2394, which lacks HMGR activity. The two *Cm-HMGR*-containing yeast strains HMG-Y4311 and HMG-Y4312 were selected on minimal medium containing galactose, and their growth was compared to that of JRY2394 and of the same strain carrying only the empty-vector (pYES2) (Fig. 3). All four strains were unable to grow on glucose-supplemented YM, but HMG-Y4311 and HMG-Y4312 grew on galactose-supplemented YM. In addition to the two *Cm-HMGR*-containing strains, the pYES2-transformed strain grew on YM supplemented with galactose and mevalonate; further addition of uracil enabled all four strains to grow.

Northern analysis confirmed that galactose strongly induced transcription of *Cm-HMGR* in HMG-Y4311 and HMG-Y4312 (Table 1). The HMGR activity assay showed that HMG-Y4311 and HMG-Y4312 produce functional HMGR. Both transgenic yeast lines had negligible levels of *Cm-HMGR* mRNA before induction, the levels of HMGR activity were markedly low, and these uninduced lines failed to grow in the absence of added mevalonate (data not shown). These results indicate that *Cm-HMGR* mRNA transcribed from the cloned cDNA insert encodes a functional HMGR protein.

Genomic Southern analysis

We used genomic Southern analysis to evaluate the genomic organization of *Cm-HMGR* in Fuyu A, Natsu 4, and their F1 hybrid. At high stringency, each of these samples was associated with a single, strong signal. At a low stringency, two weaker signals were found, in addition to the strong band in these lanes (Fig. 4). These results suggest that *Cm-HMGR* belongs to a small family of *hmgr* genes. In addition, the *Cm-HMGR*

Fig. 3 Complementation for growth of the yeast strain JRY2394, which lacks HMGR activity, by the *Cm-HMGR* cDNA. Growth of HMG-Y4311 and HMG-Y4312, which express Cm-HMGR, and the strain transformed with pYES2 is shown. For selection, the medium was supplemented with galactose to induce production of Cm-HMGR. The non-selective medium contained mevalonate and uracil

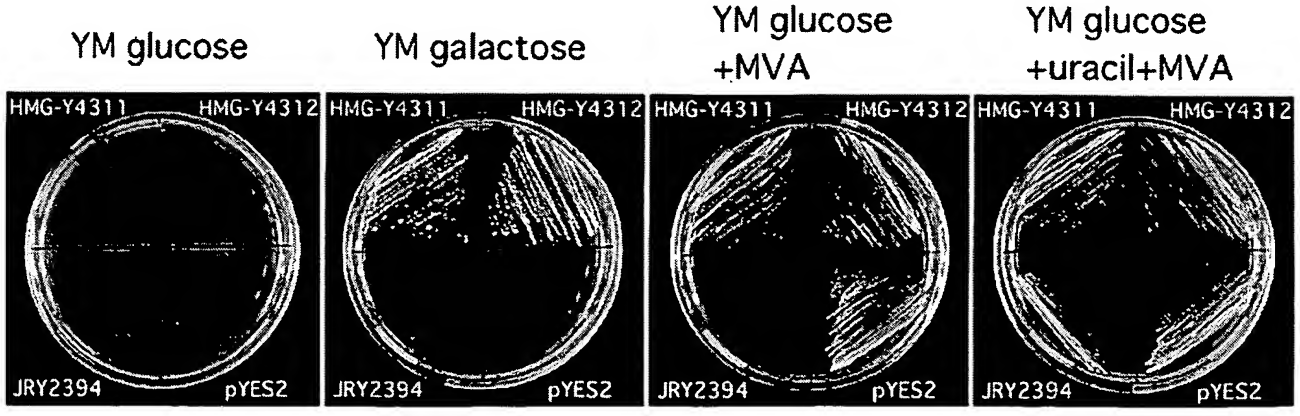

Table 1 HMGR activity of yeast lines transformed with *Cm-HMGR*

Yeast line	Medium	Plasmid	HMGR activity[a]	*HMGR* signal on Northern blot
HMG-Y4311	YM galactose	pYES::Cm-HMGR	68.98	Strong
HMG-Y4311	YM glucose + MVA	pYES::Cm-HMGR	0.09	Weak
HMG-Y4312	YM galactose	pYES::Cm-HMGR	56.28	Strong
HMG-Y4312	YM glucose + MVA	pYES::Cm-HMGR	0.09	Weak
JRY2394	YM glucose + MVA + Ura	pYES2	0.00	Not done
TM100	YM glucose + Ura + Lu - Trp	Not applicable	49.26	Not done

[a] HMGR activity is expressed in nmols of mevalonate (MVA) produced per h per mg HMGR protein

cDNA probe hybridized only with *Cm-HMGR* mRNA under high-stringency washing conditions.

Cm-HMGR in developing melon fruits

Fruit weights of Fuyu A, Natsu 4, and the hybrid Fuyu A × Natsu 4 (Fig. 5A) increased after pollination. The pattern of increasing fruit weight in Fuyu A fitted the logarithmic curve $y = 1341.0 \log(x) - 597.1$ ($r^2 = 0.84$); the equivalent curves for Natsu 4 and the F1 hybrid are given by the equations $y = 750.9 \log(x) - 316.9$ ($r^2 = 0.84$) and $y = 1070.8 \log(x) - 473.1$ ($r^2 = 0.85$), respectively. The fruit size of Fuyu A was larger than that of Natsu 4, and that of the hybrid was intermediate between Fuyu A and Natsu 4. These results suggest that the differences in fruit weight were controlled by genetic factors.

We used RNA blot hybridization to detect changes in the accumulation of *Cm-HMGR* transcripts during fruit development (Fig. 5B, D). During early fruit development in all three genotypes, *Cm-HMGR* transcripts accumulated rapidly during the first week after pollination, after which they decreased until reaching a minimum at 21 days after pollination. During the middle and late stages of fruit development (21 days after pollination and later), levels of *Cm-HMGR* transcripts in Fuyu A increased markedly, whereas in Natsu 4 and the hybrid they increased only slightly. More *Cm-HMGR* transcripts accumulated in the fruit of Fuyu A than in Natsu 4; the quantity of *Cm-HMGR* mRNA in the hybrid was intermediate between that of Fuyu A and Natsu 4 throughout fruit development.

We assayed HMGR activity during fruit development in the three melon genotypes (Fig. 5C). For all three genotypes, HMGR activity increased rapidly during the first week after pollination, then decreased until 21 days after pollination, by which time the activity was undetectable. HMGR activity in the fruit of Fuyu A was higher than that in Natsu 4, whereas that in the hybrid was intermediate between Fuyu A and Natsu 4 throughout fruit development.

Discussion

After cloning the cDNA from the developing fruit of the melon *Cucumis melo* L. *reticulutus*, we characterized *Cm-HMGR* through complementation of the *HMGR*-deficient yeast strain JRY2394 and analyzed transcription levels and HMGR activity during fruit development. We obtained *Cm-HMGR* by chance while screening a cDNA library with a fragment of the gene

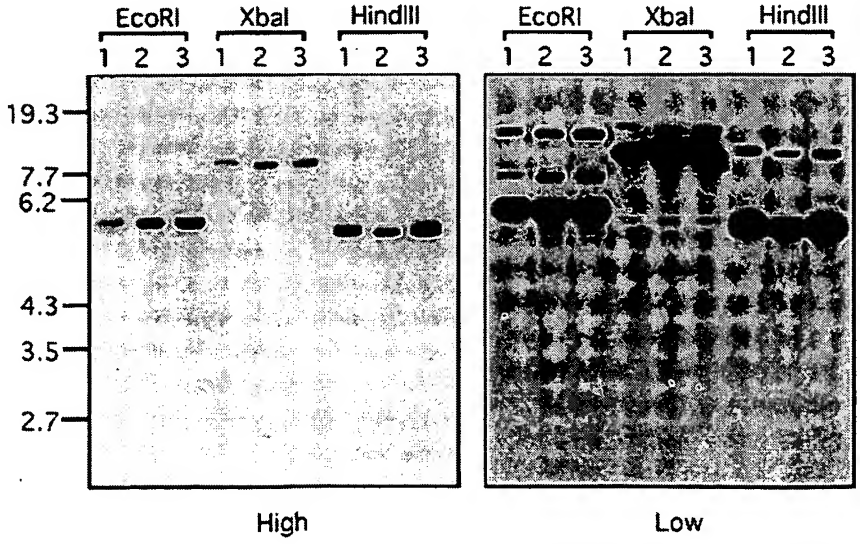

Fig. 4 Southern analysis of melon genomic DNA probed with the 1.25-kb *Cm-HMGR* cDNA fragment encoding the catalytic domains. Digested genomic DNA was electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane. The filter was first hybridized overnight at low stringency (60 °C, Low) and exposed to X-ray film for 4 days. The membrane was then stripped, reprobed overnight at high stringency (65 °C, High), and exposed to film for 2 days. Molecular size markers are indicated on the left. Lanes 1, Fuyu A; 2, Fuyu A × Natsu 4 hybrid; 3, Natsu 4

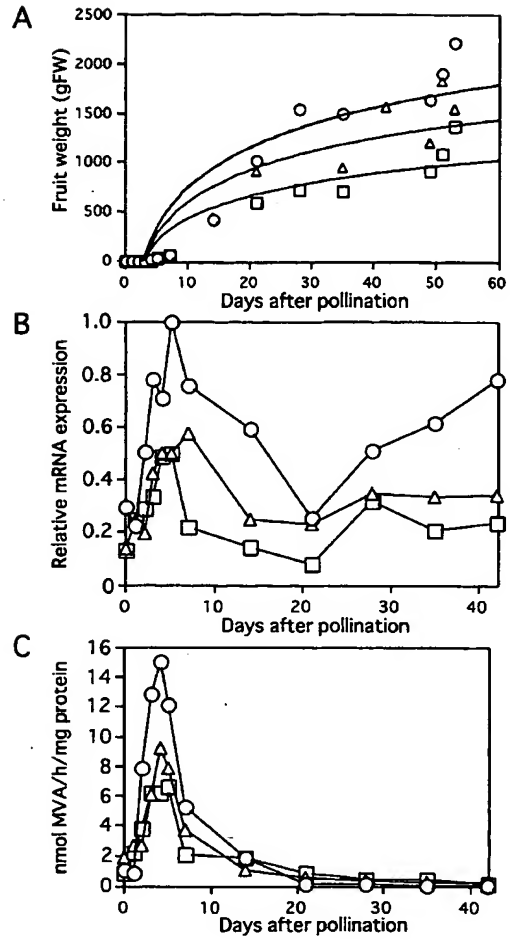

Fig. 5A–D Comparison of fruit growth, HMGR activity, and transcription of *Cm-HMGR* in the melon cultivars Fuyu A and Natsu 4 and the hybrid Fuyu A × Natus 4. **A** Fruit growth after pollination. **B** Relative levels of *Cm-HMGR* mRNA transcripts after pollination. Results are expressed as a percentage of rRNA transcripts. **C** HMGR activity in fruit after pollination. **D** Northern analysis of *Cm-HMGR*. Total RNA was isolated from fruits of each cultivar at 0, 1, 2, 3, 4, 5, 7, 14, 21, 28, 35 and 42 days after pollination. Northern analysis was performed using 10 µg of total RNA per lane and the sample was first probed with the 1.25-kb *Sal*I-*Bgl*II fragment encoding the catalytic domain of Cm-HMGR. After hybridization the membrane was washed and exposed to film. The probe was then removed and the sample was reprobed with an rRNA probe to control for loading

for the melon ethylene receptor, *Cm-ERS1* (Sato-Nara et al. 1999). The degree of homology between *Cm-HMGR* and *Cm-ERS1* is low. However, the high frequency of *Cm-HMGR* transcripts in the cDNA library, which was prepared from fruit tissue, may account for our retrieval of the *Cm-HMGR* cDNA during the screen.

Two peaks of *Cm-HMGR* mRNA accumulation occurred during melon fruit development. The first peak is observed during early fruit development, the other at ripening. *HMGR* mRNA accumulation follows this same pattern during tomato fruit development (Narita and Gruissem 1989; Park et al. 1992), but these peaks are due to differential transcription of two *HMGR* genes, *HMG1* (transcripts of which peaked early during fruit development) and *HMG2* (which is responsible for the ripening-associated peak). The phylogenetic tree of plant HMGRs (Fig. 2) shows that Cm-HMGR and tomato HMG2 belong to different groups; therefore, Cm-HMGR may be a homolog of tomato HMG1. However, characterization of the promoter regions of tomato *HMG1* and *HMG2* (Draselia et al. 1996; Jelesko et al. 1999) suggests that *HMG2* is a gene that evolved late for a specialized function in tomato, rather than playing a role in cell division (Draselia et al. 1996). These results suggest that during fruit development, the transcription of *Cm-HMGR*, the only *HMGR* gene in melon, is regulated similarly to that of the two genes, *HMG1* and *HMG2*, in tomato. Comparison of the promoter region of *Cm-HMGR* with those of tomato *HMG1* and *HMG2* will perhaps provide new insights into the evolution of *HMGR* genes in plants.

Although *Cm-HMGR* mRNA transcripts were expressed during early fruit development and ripening, HMGR activity was associated only with fruit development. Several studies have reported increased HMGR activity which reflect increases in the abundance of HMGR mRNA (Yang et al. 1991; Chye et al. 1992). One possible explanation for our observation is translational regulation of Cm-HMGR during ripening; confirmation of this hypothesis will require a specific antibody against Cm-HMGR. Another possible explanation is post-translational regulation that inactivates Cm-HMGR during fruit ripening (assuming that the protein is actually synthesized during this stage). Reversible phosphorylation, calcium, proteolytic degradation, and subcellular compartmentation all regulate HMGR activity in plants (Stermer et al. 1994); further studies will be needed to determine whether one

of these factors inactivates Cm-HMGR during fruit ripening. Interestingly, HMGR activity and *hmg1* transcription in tomato fruit was highest during early fruit development; however, HMGR activity in ripening tomato fruits was low even though the level of *hmg2* transcription was high (Narita and Gruissem 1989). Together, these results suggest that a common mechanism exists and accounts for the inactivation of HMGR during fruit ripening.

How does *Cm-HMGR* relate to melon fruit development? As in most plants, fruit development in melon is divided into three phases (Gillaspy et al. 1993). Phase I involves ovary development, fertilization, and fruit set. During phase II, fruit growth is primarily due to cell division, whereas growth during phase III occurs mainly by cell expansion and is followed by ripening. We found that *Cm-HMGR* transcription and HMGR activity were markedly increased after melon fruit set, which is coincident with phase II of fruit development. A similar pattern of *HMG1* mRNA accumulation and HMGR activity is observed during the early stage of tomato fruit development (Narita and Gruissem 1989). HMGR catalyzes the conversion of HMG-CoA into mevalonate (MVA), which is the precursor of phytosterols (Gillaspy et al. 1993). Since HMGR is an important control point for the mevalonate pathway in plants (Maurey et al. 1986), the increase in HMGR should result in increasing the supply of phytosterol. In addition, sterols are major components of the eukaryotic cell membrane, and are required for the production of cell membrane, which is a necessary step in cell division. Therefore, *Cm-HMGR* is likely to be involved in cell division during phase II after melon fruit set.

The amount of *Cm-HMGR* mRNA in the fruit of Fuyu A was higher than that in Natsu 4 whereas that in hybrid Fuyu A × Natsu 4 was intermediate between those of the parents; the pattern of accumulation of transcripts seemed to be similar. The level of *Cm-HMGR* mRNA accumulation in the fruit of the three genotypes was associated with fruit size. Interestingly, HMGR activity also correlated with fruit size in melon. In comparison with normal and small-fruit cultivars of avocado, HMGR activities in mesocarp are also associated with cell number and fruit size (Cowan et al. 1997). The amount of cell proliferation during early fruit development determines fruit size in melon (Higashi et al. 1999). Together, these results suggest that by stimulating cell division, *Cm-HMGR* is involved in regulating fruit size in these melon genotypes.

Our results show that *Cm-HMGR* is involved in early events of melon fruit development, most likely cell proliferation after pollination. Transcription levels of *HMG1* in tomato (Narita and Gruissem 1989) and physiological analysis of avocado by using a competitive inhibitor of HMGR (Cowan et al. 1997) point to the importance of HMGR for fruit development in these plants. Further understanding of the role of *Cm-HMGR* in melon fruit development requires direct evidence of the functional connection between cell division and HMGR activity. In addition, analysis of transgenic fruit that expresses Cm-HMGR will provide useful information regarding the association between this protein and fruit development in melon.

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Genetics

3-Hydroxy-3-methylglutaryl-coenzyme A reductase from *Arabidopsis thaliana* is structurally distinct from the yeast and animal enzymes

(isoprenoid biosynthesis/plant/suppression)

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Contributed by Gerald R. Fink, January 18, 1989

ABSTRACT **We have isolated the *Arabidopsis thaliana* gene (*HMG1*) encoding 3-hydroxy-3-methylglutaryl-CoA reductase [HMG-CoA reductase; (*S*)-mevalonate:NAD$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.88], the catalyst of the first committed step in isoprenoid biosynthesis. cDNA copies of the plant gene were identified by hybridization with a short, highly conserved segment of yeast HMG-CoA reductase as probe. DNA sequence analysis reveals that the COOH-terminal domain of the *Arabidopsis* HMG-CoA reductase (containing the catalytic site of the enzyme) is highly conserved with respect to the yeast, mammalian, and *Drosophila* enzymes, whereas the membrane-bound amino terminus of the *Arabidopsis* protein is truncated and lacks the complex membrane-spanning architecture of the yeast and animal reductases. Expression of the *Arabidopsis* gene from the yeast *GAL1* promoter in a yeast mutant lacking HMG-CoA reductase activity suppresses the growth defect of the yeast mutant. Taken together, the sequence similarity to other cloned HMG-CoA reductase genes and the suppression of the yeast *hmg*$^-$ mutant provide strong evidence that the novel *Arabidopsis* gene we have cloned encodes a functional HMG-CoA reductase enzyme.**

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [(*S*)-mevalonate:NAD$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.88] catalyzes the first committed step in the isoprenoid biosynthetic pathway, the synthesis of mevalonic acid (MVA) from HMG-CoA. MVA is precursor to the five-carbon isoprene unit that serves as the fundamental building block for a number of biologically important compounds, including sterols, dolichol, ubiquinone, and isopentylated adenine. In mammals, the multivalent regulation of HMG-CoA reductase (1) provides a sensitive control mechanism that responds to the levels of serum cholesterol and coordinates isoprenoid metabolism to ensure the availability of both sterol and nonsterol products.

The plant isoprenoid biosynthetic pathway has numerous additional branches that give rise to a number of unique products, including growth regulators (such as cytokinin, gibberellin, and abscisic acid), photosynthetic pigments, phytotoxins, phytoalexins, and a variety of specialized terpenoids. The regulated synthesis of these isoprenoid compounds is essential to plant growth and development. Studies of HMG-CoA reductase regulation in plants suggest that the enzyme activity responds to a variety of external stimuli, including light (2–4), plant growth regulators (3, 5, 6), sterols (5), and wounding and plant pathogens (7, 8). Furthermore, the levels of HMG-CoA reductase activity vary markedly at different stages of development and in different plant tissues (9). Whereas the mammalian and yeast enzymes appear to be localized in the cytoplasm (10, 12), plant HMG-CoA reductase has been found in mitochondria and plastids as well as the microsomal fractions in some, but not all, species (2–8, 13). These studies raise the possibility that independent isoprenoid pathways operate in each of the compartments (14). However, the membrane association of the protein makes these determinations difficult; thus, the studies of plant HMG-CoA reductase have been impeded by the absence of adequate molecular probes. We have cloned the HMG-CoA reductase gene of *Arabidopsis thaliana*, *HMG1*, as a first step in characterizing the temporal and spatial regulation of this enzyme in higher plants.

MATERIALS AND METHODS

Library Screening. Plaques (50,000) from an *Arabidopsis* cDNA library in phage λ gt10 were transferred to nylon filters (15). A 283-base-pair (bp) *Hin*dIII/*Bst*NI fragment, containing the most highly conserved sequences between the yeast and hamster HMG-CoA reductase genes (16), was used to probe the nylon membranes under conditions of low-stringency hybridization in solutions containing 25% formamide (17, 23). Genomic clones were isolated from a phage λ EMBL4 library containing *Arabidopsis* DNA inserts by using fragments of the *Arabidopsis* HMG-CoA reductase cDNA clone as a probe. Hybridization and washing were carried out under conditions of high stringency in solutions containing 50% formamide (23). Phage yielding a positive signal on duplicate filters were purified, the *Eco*RI insert fragments that hybridized to the reductase probes were isolated and subcloned into pUC119 (18), and the recombinant plasmids were propagated in *Escherichia coli* strain JM109 (19).

DNA Sequencing. Nested deletions in the insert fragment were generated by digestion with *Exo* III by the method of Henikoff (20) with modifications. Single-stranded DNA was prepared as described (18) and sequenced by the dideoxy chain-termination method (21).

Preparation of *Arabidopsis* RNA. The Columbia strain of *Arabidopsis thaliana* (L. Heynh) was grown under continuous light at 22°C, and RNA was prepared from whole plants by the sodium dodecyl sulfate/phenol procedure (23). Poly(A)$^+$ fractions of RNA were selected by chromatography on oligo(dT)-cellulose (24).

Yeast Molecular Genetics. Yeast media were prepared as described (25). The full-length *Arabidopsis* HMG1 cDNA was placed under the control of the yeast *GAL1* promoter in the vector pCG5109 (a gift of Collaborative Research) by inserting the 2.4-kb *Bam*HI–*Sal* I fragment from pUCHMG2 into the unique *Bam*HI site in the GAL1 RNA leader to generate pYHMG1. Translation of the HMG-CoA reductase RNA initiates at the first ATG codon in the *Arabidopsis* gene. The recipient for heterologous expression of *Arabidopsis HMG1* was a derivative of the yeast strain JRY1145, a *hmg1::LYS2 hmg2::HIS3 lys2-801 his3Δ200 ura3-52 ade2-101 met* pJR401 (26), which had been cured of pJR401

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonic acid.

(containing the yeast *HMG2* gene) by selection on yeast extract/peptone/dextrose containing 5 mg of mevalonic acid per ml and 1% 5-fluoroorotic acid (27) to generate the MVA auxotroph. Plasmids containing the yeast *URA3* gene were introduced into this yeast strain by the lithium transformation method of Ito *et al.* (28), and transformants were selected on the basis of their ability to grow in the absence of uracil.

RESULTS

Isolation and DNA Sequence Analysis of the *Arabidopsis* HMG-CoA Reductase Gene, *HMG1*. Our strategy for isolating the gene for HMG-CoA reductase from *Arabidopsis* was based on the observation that the genes from hamster and yeast contained highly conserved regions (16). We used a short fragment from yeast *HMG1* (nucleotides +2324 to +2607, relative to the start site of translation) to identify cDNA copies of the *Arabidopsis* gene. Preliminary sequence analysis of three *Arabidopsis* cDNA clones identified by this method revealed extended regions of sequence similarity with yeast *HMG1*, suggesting that we had isolated a portion of the *Arabidopsis* HMG-CoA reductase gene. To obtain the entire coding sequence of the gene as well as flanking sequences, we used the *Arabidopsis* cDNA clone to isolate genomic clones from an *Arabidopsis* library. Restriction analysis of nine cross-hybridizing phage revealed that all of the clones were overlapping, having in common both a 5.0- and 2.8-kilobase (kb) *Eco*RI fragment that hybridized to the *Arabidopsis* cDNA probe (data not shown).

We determined the complete DNA sequence of all three cDNA clones and 7.8 kb of genomic DNA from *Arabidopsis*.* The amino acid sequence of the *Arabidopsis* HMG-CoA reductase cDNA deduced from the DNA sequence (Fig. 1) reveals an open reading frame of 592 amino acids encoding a protein with a calculated molecular mass of 75,785 Da. The reading frame in the genomic DNA is interrupted by three introns (Fig. 1), as inferred from the contiguous coding sequence of the cDNA and confirmed by S1 nuclease mapping (data not shown).

Hybridization of the *Arabidopsis* HMG1 cDNA probe to *Arabidopsis* genomic DNA revealed two *Eco*RI fragments identical in size (5.0 and 2.8 kb) to the cloned *Eco*RI fragments isolated from the genomic library (data not shown). Moreover, the pattern of genomic restriction fragments that hybridize to the cDNA probe is entirely consistent with predictions based on the nucleotide sequence of the genomic DNA, confirming the *Arabidopsis* origin of the cloned gene.

Analysis of the Predicted Amino Acid Sequence. The predicted amino acid sequence from the *Arabidopsis HMG1* gene was aligned with the sequence predicted from the yeast *HMG1* gene (16) to optimize their sequence similarity, revealing a number of unique features of the *Arabidopsis* HMG-CoA reductase protein (Fig. 2*A*). First, *Arabidopsis HMG1* encodes a much smaller polypeptide (592 amino acids) than either the yeast (1050 amino acids) (16), hamster (871 amino acids) (30), or *Drosophila* genes (916 amino acids) (31). Second, the amino acid residues that are conserved between the yeast and *Arabidopsis* enzymes are confined largely to the COOH-terminal half of the proteins (Fig. 2*A*). Amino acids 21–592 of the *Arabidopsis HMG1*-encoded polypeptide are identical at 42% of the positions and show conservative changes at 19% of the positions when aligned with amino acids 473–1054 of the yeast *HMG1*-encoded protein. Models for the structure of the yeast, mammalian, and *Drosophila* enzymes divide the protein into two domains (16, 31, 32). The hydrophilic, cytoplasmic COOH-terminal domain contains the active site (32), and sequences in this domain have been

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04537).

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-75 ACTTATCACGCCACCTCACCACCTCTCTCCTACTCTCCTCTCTCT -31
-30 CCCCCCTGGAGAGATTATTCATTCCCTCCAATGGATCTCCGTCGG +15
 +1                          M  D  L  R  R           5
+16 AGGCCTCCTAAACCACCGGTTACCAACAACAACAACTCCAACGGA  60
  6 R  P  P  K  P  P  V  T  N  N  N  N  S  N  G    20
 61 TCTTTCCGTTCTTATCAGCCTCGCACTTCCGATGACGATCATCGT 105
 21 S  F  R  S  Y  Q  P  R  T  S  D  D  D  H  R    35
106 CGCCGGGCTACAACAATTGCTCCTCCACCGAAAGCATCCGACGCG 150
 36 R  R  A  T  T  I  A  P  P  P  K  A  S  D  A    50
151 CTTCCTCTTCCGTTATATCTCACAAACGCCGTTTTCTTCACGCTC 195
 51 L  P  L  P  L  Y  L  T  N  A  V  F  F  T  L    65
196 TTCTTCTCCGTCGCGTATTACCTCCTCCACCGGTGGCGTGACAAG 240
 66 F  F  S  V  A  Y  Y  L  L  H  R  W  R  D  K    80
241 ATCCGTTACAATACGCCTCTTCACGTCGTCACTATCACAGAACTC 285
 81 I  R  Y  N  T  P  L  H  V  V  T  I  T  E  L    95
286 GGCGCCATTATTGCTCTCATCGCTTCGTTTATCTATCTCCTAGGG 330
 96 G  A  I  I  A  L  I  A  S  F  I  Y  L  L  G   110
331 TTTTTGGTATTGACTTTGTTCAGTCATTTATCTCACGTGCCTCT 375
111 F  F  G  I  D  F  V  Q  S  F  I  S  R  A  S   125
376 GGTGATGCTTGGGATCTCGCCGATACGATCGATGATGATGACCAC 420
126 G  D  A  N  D  L  A  D  T  I  D  D  D  D  H   140
421 CGCCTTGTCACGTGCTCTCCACCGACTCCGATCGTTTCCGTTGCT 465
141 R  L  V  T  C  S  P  P  T  P  I  V  S  V  A   155
466 AAATTACCTAATCCGGAACCTATTGTTACCGAATCGCTTCCTGAG 510
156 K  L  P  N  P  E  P  I  V  T  E  S  L  P  E   170
511 GAAGACGAGGAGATTGTGAAATCGGTTATCGACGGAGTTATTCCA 555
171 E  D  E  E  I  V  K  S  V  I  D  G  V  I  P   185
556 TCGTACTCGCTTGAATCTCGTCTCGGTGATTGCAAAAGAGCGGCG 600
186 S  Y  S  L  E  S  R  L  G  D  C  K  R  A  A   200
601 TCGATTCGTCGTGAGGCGTTGCAGAGAGTCACCGGGAGATCGATT 645
196 S  I  R  R  E  A  L  Q  R  V  T  G  R  S  I   215
646 GAAGGGTTACCGTTGGATGGATTTGATTATGAATCGATTTTGGGG 690
216 E  G  L  P  L  D  G  F  D  Y  E  S  I  L  G   230
691 CAATGCTGTGAGATGCCTGTTGGATACATTCAGATTCCTGTTGGG 735
231 Q  C  C  E  M  P  V  G  Y  I  Q  I  P  V  G   245
736 ATTGCTGGTCCATTGTTGCTTGATGGTTATGAGTACTCTGTTCCT 780
246 I  A  G  P  L  L  D  G  Y  E  Y  S  V  P      260
781 ATGGCTACAACCGAAGGTTGTTTGGTTGCTAGCACTAACAGAGGC 825
261 M  A  T  T  E  G  C  L  V  A  S  T  N  R  G   275
826 TGCAAGGCTATGTTTATCTCTGGTGGCGCCACCAGTACCGTTCTT 870
276 C  K  A  M  F  I  S  G  G  A  T  S  T  V  L   290
871 AAGGACGGTATGACCCGAGCACCTGTTGTTCGGTTCGCTTCGGCG 915
291 K  D  G  M  T  R  A  P  V  V  R  F  A  S  A   305
916 AGACGAGCTTCGGAGCTTAAGTTTTCTTGGAGAATCCAGAGAAC 960
306 R  R  A  S  E  L  K  F  F  L  E  N  P  E  N   320
961 TTTGATACTTTGGCAGTAGTCTTCAACAGGTCGAGTAGATTTGCA 1005
321 F  D  T  L  A  V  V  F  N  R  S  S  R  F  A   335
1006 AGACTGCAAAGTGTTAAATGCACAATCGCGGGGAAGAATGCTTAT 1050
 336 R  L  Q  S  V  K  C  T  I  A  G  K  N  A  Y   350
1051 GTAAGGTTCTGTTGTAGTACTGGTGATGCTATGGGGATGAATATG 1095
 351 V  R  F  C  C  S  T  G  D  A  M  G  M  N  M   365
1096 GTTTCTAAAGGTGTGCAGAATGTTCTTGAGTATCTTACCGATGAT 1140
 366 V  S  K  G  V  Q  N  V  L  E  Y  L  T  D  D   380
1141 TTCCCTGACATGGATGTGATTGGAATCTCTGGTAACTTCTGTTCG 1185
 381 F  P  D  M  D  V  I  G  I  S  G  N  F  C  S   395
1186 GACAAGAAACCTGCTGCTGTGAACTGGATTGAGGGACGTGGTAAA 1230
 396 D  K  K  P  A  A  V  N  W  I  E  G  R  G  K   410
1231 TCAGTTGTTTGCGAGGCTGTAATCAGAGGAGAGATCGTGAACAAG 1275
 411 S  V  V  C  E  A  V  I  R  G  E  I  V  N  K   425
1276 GTCTTGAAAACGAGCGTGGCTGCTTTAGTCGAGCTCAACATGCTC 1320
 426 V  L  K  T  S  V  A  A  L  V  E  L  N  M  L   440
1321 AAGAACCTAGCTGGCTCTGCTGTTGCAGGCTCTCTAGGTGGATTC 1365
 441 K  N  L  A  G  S  A  V  A  G  S  L  G  G  F   455
1366 AACGCTCATGCCAGTAACATAGTGTCTGCTGTATTCATAGCTACT 1410
 456 N  A  H  A  S  N  I  V  S  A  V  F  I  A  T   470
1411 GGCCAAGATCCAGCTCAAAACGTGGAGAGTTCTCAATGCATCACC 1455
 456 G  Q  D  P  A  Q  N  V  E  S  S  Q  C  I  T   485
1456 ATGATGGAAGCTATTAATGACGGCAAAGATATCCATATCTCAGTC 1500
 486 M  M  E  A  I  N  D  G  K  D  I  H  I  S  V   500
1501 ACTATGCCATCTATCGAGGTGGGGACAGTGGGAGGAGGAACACAG 1545
 501 T  M  P  S  I  E  V  G  T  V  G  G  G  T  Q   515
1546 CTTGCATCTCAATCAGCGTGTTTAAACCTGCTCGGAGTTAAAGGA 1590
 516 L  A  S  Q  S  A  C  L  N  L  L  G  V  K  G   530
1591 GCAAGCACAGAGTCGCCGGGAATGAACGCAAGGAGGCTAGCGACG 1635
 531 A  S  T  E  S  P  G  M  N  A  R  R  L  A  T   545
1636 ATCGTAGCCGGAGCAGTTTAGCTGGAGAGTTATCTTTAATGTCA 1680
 546 I  V  A  G  A  V  L  A  G  E  L  S  L  M  S   560
1681 GCAATTGCAGCTGGACAGCTTGTGAGAAGTCACATGAAATACAAT 1725
 561 A  I  A  A  G  Q  L  V  R  S  H  M  K  Y  N   575
1726 AGATCCAGCCGAGACATCTCTGGAGCAACGACAACGACAACAACA 1770
 576 R  S  S  R  D  I  S  G  A  T  T  T  T  T  T   590
1771 ACAACATGATCTGAATCTGAATCATCATCCTCTCAAAGAAGGACA 1815
 591 T  T  *                                       592
1816 ACAATCCAAAAACAAGGGCAGGCTTTTTACAACGCATTCCAACCTC 1860
1861 AAAACTCGCTGGTGGACAGATTTTAGCCATGGTGGCGGTAATGCG 1905
1906 TTTGCCCTTTTGTTAAAATAAAAAAACTATTTGTTTTGTTTGTTT 1950
1951 GACTTGATATCTTTTTTTGGGATTGAGGATTGAGAGAGATAGAGA 1995
1996 GATTTTACAAACTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCATG 2040
2041 GATAATTCGTGTCTCTTTGATTTGTCTAAGGTTTGTCTTTGTTTG 2085
2086 TTAGGAAGTGGTCTATATGAACGAAAAATTTGTGT             2120
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FIG. 1. Nucleotide sequence of *Arabidopsis* HMG-CoA reductase gene *HMG1* and the deduced amino acid sequence. Nucleotide or amino acid residues (single-letter code in italics) are numbered relative to the ATG (+1) that initiates the first open reading frame in the coding sequence. Nucleotides that are underlined designate the position of intervening sequences in the gene. The sizes of the introns in nucleotides (nt) are as follows: I, 223 nt; II, 159 nt; III, 91 nt.

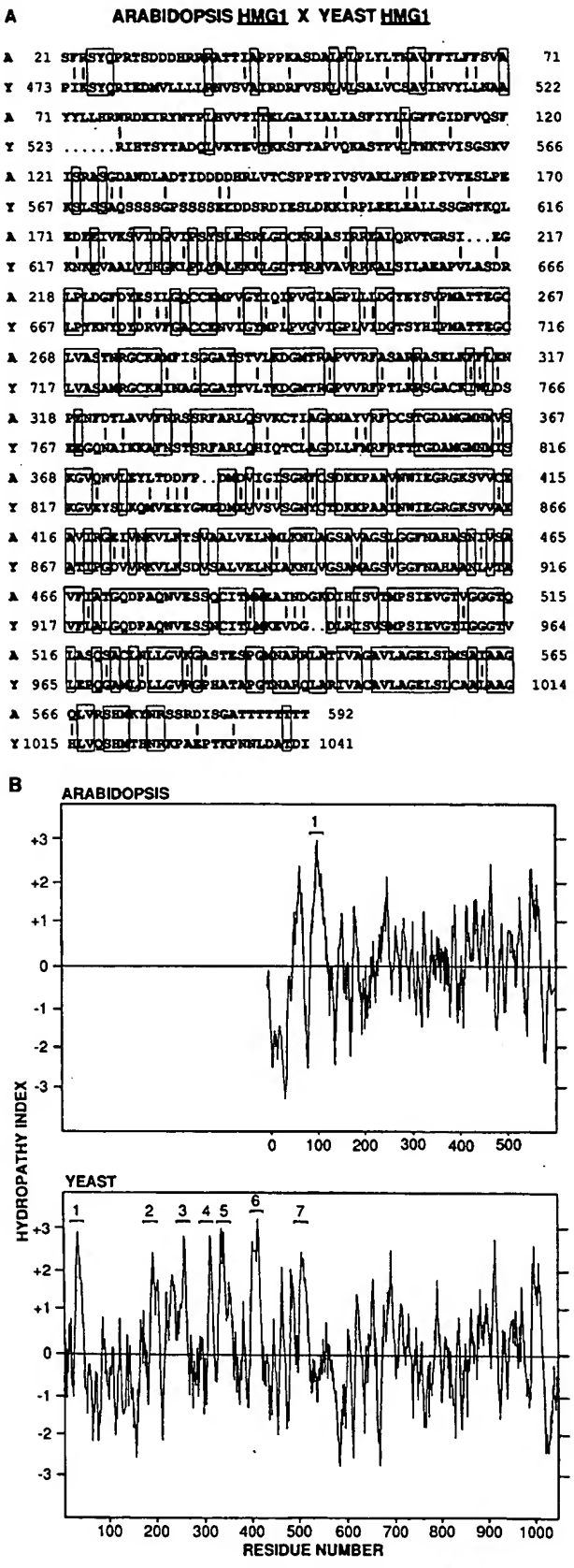

FIG. 2. Comparison of the *Arabidopsis* and yeast HMG-CoA reductase proteins. (*A*) Identical residues in the two proteins are enclosed by boxes: *Arabidopsis* lines A; yeast (16), lines Y. Conservative amino acid changes are designated by vertical lines between the *Arabidopsis* and yeast residues. No sequence similarity between yeast and *Arabidopsis* HMG-CoA reductases was detected prior to amino acid residue 473 in the yeast protein. (*B*) Hydropathy plots of yeast and *Arabidopsis* HMG-CoA reductase proteins. The average hydrophobicity of each amino acid residue was calculated by the method of Kyte and Doolittle (29) over a window of nine amino acids and was plotted as a function of amino acid position. The graphs were aligned to maximize structural similarities. The labeled peaks indicate the membrane-spanning regions in the amino-terminal domain of the proteins. The hydropathy plot of yeast *HMG1* was replotted from Basson *et al.* (16).

highly conserved between the animal, fungal, and plant HMG-CoA reductases.

The amino-terminal domain of all the cloned reductases (residues 1–339 in hamster, 1–525 in yeast, and 1–380 in *Drosophila*) are predicted to contain seven hydrophobic transmembrane-spanning segments (Fig. 2*B*) thought to anchor the enzyme to the endoplasmic reticulum (33). Despite this conservation of secondary structure (16), the sequences in this domain have not been conserved between yeast and the higher eukaryotes. Although plant HMG-CoA reductase also appears to be an integral membrane protein of the endoplasmic reticulum (see refs. 13 and 14 for review), not only has the sequence of the amino-terminal domain of *Arabidopsis* HMG-CoA reductase diverged completely from that of yeast (Fig. 2*A*), but also a much simpler structure for the amino-terminal anchor can be predicted (Fig. 2*B*). Based on the algorithm of Klein *et al.* (34), *Arabidopsis* HMG-CoA reductase is predicted to have only a single membrane-spanning helix in the amino-terminal domain, including amino acid residues 86–118.

These primary and secondary structural considerations lead to the following conclusion: the amino acid sequence of the COOH-terminal domain of the *Arabidopsis* HMG-CoA reductase (containing the catalytic site of the enzyme) is highly conserved with respect to yeast and other eukaryotic reductases, whereas the membrane-bound amino terminus of the *Arabidopsis* protein is truncated, lacking the complex membrane-spanning architecture of the yeast, mammalian, and *Drosophila* enzymes.

Analysis of *Arabidopsis* HMG-CoA Reductase RNA. RNA blot-hybridization analysis reveals a single RNA species when hybridized to the *Arabidopsis* HMG1 cDNA probe. This 2300-nucleotide transcript is present in preparations of both total (Fig. 3*A*, lanes 1 and 2) and poly(A)$^+$-selected RNA (Fig. 3*A*, lanes 3 and 4).

Primer extension and ribonuclease protection analyses were performed to identify the 5′ end of the reductase mRNA. A number of discrete reaction products were detected both by primer extension (Fig. 3*B*, lane 6) and by ribonuclease protection (Fig. 3*C*, lane 2). The 5′ termini of all of these transcripts map to positions between 68 and 74 nucleotides upstream of the putative ATG codon for translation initiation. The additional microheterogeneity in the 5′ ends of the RNA observed when using RNase protection may be attributed to digestion artifacts inherent with the assay.

***Arabidopsis* HMG-CoA Reductase Functions in an *hmg*$^-$ Yeast Strain.** We tested whether the gene we have identified as *Arabidopsis* HMG-CoA reductase (*HMG1*) encodes this enzymatic activity by determining whether our clone can suppress the HMG-CoA reductase deficiency in a yeast mutant (JRY1145) that lacks both HMG-CoA reductase isozymes (*hmg1 hmg2*) (26). These mutations result in a growth defect on standard yeast minimal medium that can be bypassed by high concentrations of MVA, the product of the reaction catalyzed by reductase. In our experiment, expression of the *Arabidopsis HMG1* gene is under the control of the yeast *GAL1* promoter, which is transcriptionally active in yeast cells grown in galactose and inactive in cells grown in

glucose. The *hmg1 hmg2* yeast strain transformed with the *GAL1::HMG1 (Arabidopsis)* construct was tested for growth on minimal essential medium containing either glucose or galactose.

The double-mutant *hmg1 hmg2* yeast strain harboring *Arabidopsis HMG1* grows vigorously on minimal essential medium with galactose but not with glucose (Fig. 4 *B* and *C*). That this galactose-dependent growth results from expression of *Arabidopsis HMG1* is supported by the analysis of other constructions. JRY1145, the parent *hmg1 hmg2* yeast strain, does not grow on medium with galactose or glucose unless MVA is added (Fig. 4*A*). When this same strain is transformed with a plasmid encoding yeast *HMG2* under the control of its own promoter (pJR401), the cells grow without added MVA on both galactose and glucose (Fig. 4 *B* and *C*). The ability of the plant gene to suppress a yeast strain known to be defective in HMG-CoA reductase provides strong evidence that the *Arabidopsis* cDNA encodes a functional HMG-CoA reductase enzyme.

DISCUSSION

We have isolated the gene for HMG-CoA reductase from *Arabidopsis thaliana*, using a heterologous yeast probe. The carboxyl-terminal region of the polypeptide, containing the active site of the enzyme (32), exhibits extensive sequence identity with mammalian, yeast, and *Drosophila* HMG-CoA reductases. Moreover, the expression of *Arabidopsis HMG1* in a strain of yeast lacking HMG-CoA reductase activity (26) alleviates the requirement for MVA in the yeast mutant. The isolation of the *Arabidopsis* cDNA, the conservation of amino acid sequence, and the ability of the cDNA to function in yeast provides compelling evidence that the DNA we have isolated represents the authentic *Arabidopsis* structural gene for HMG-CoA reductase.

The yeast, *Drosophila*, and mammalian proteins contain a complex membrane-bound amino-terminal domain that spans the endoplasmic reticulum seven times (16, 31, 32). The structural conservation of the amino terminus among these various HMG-CoA reductases, despite their sequence divergence, suggests some functional constraints. Indeed, in hamster, this amino-terminal domain has been implicated in targeting the enzyme to the endoplasmic reticulum, regulating its half-life in response to cholesterol (33) and controlling the biogenesis of internal membranes in the cell (22).

The amino terminus of the *Arabidopsis* enzyme is truncated by comparison with these other reductases, containing only a single membrane-spanning domain. The unusual structure of the *Arabidopsis* enzyme may reflect a special adaptation for plant-specific regulation or sorting. Indeed, in some plants HMG-CoA reductase activity is found not only in the endoplasmic reticulum but also in the mitochondrion and chloroplast (2–8, 13). The relatively simple architecture of the amino-terminal domain of *Arabidopsis* HMG-CoA reductase may facilitate differential targeting of the enzyme to several intracellular compartments. These compartmentalization studies were not done in *Arabidopsis*, but if HMG-CoA reductase also shows differential localization in *Arabidopsis*, then one must entertain the possibility that there are several forms of the enzyme in this plant. These isoforms could be generated by multiple reductase genes, alternative mRNA processing, or posttranslational modification.

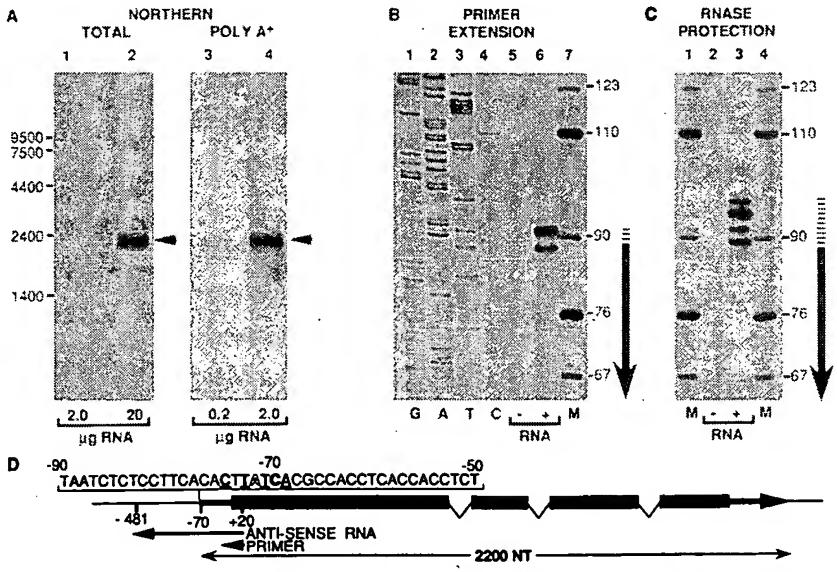

FIG. 3. Analysis of *Arabidopsis* HMG-CoA reductase mRNA. (*A*) Northern analysis. RNA was treated with glyoxal, fractionated by agarose gel electrophoresis, transferred to nitrocellulose (23), and hybridized to a full-length *Arabidopsis* HMG1 cDNA probe (17) under conditions of high stringency. The following RNA samples were assayed: 2.0 μg of total RNA (lane 1), 20 μg of total RNA (lane 2), 0.2 μg of poly(A)$^+$ RNA (lane 3), and 2.0 μg of poly(A)$^+$ RNA (lane 4). The nucleotide length of the RNAs, indicated to the left of the autoradiogram, was determined with RNA standards prepared by BRL. (*B*) Primer extension. An oligonucleotide (extending from −7 to +20) was hybridized to 50 μg of total RNA and used to prime cDNA synthesis (lane 6) (37). Primer extension was also carried out in the presence of 10 μg of yeast tRNA (lane 5). In addition, the primer was used to prepare a sequencing ladder to identify the 5′ terminus of HMG1 mRNA (lanes 1–4). (*C*) Ribonuclease protection. An antisense RNA probe (extending from −481 to +20) was synthesized *in vitro* by using phage SP6 RNA polymerase and [α-^{32}P]UTP and hybridized to 50 μg of total *Arabidopsis* RNA (lane 3) or 10 μg of yeast tRNA (lane 2); the hybrid was digested with RNase A and RNase T1 (36). In both primer extension and ribonuclease protection assays, the reaction products were fractionated on an 8% polyacrylamide gel containing 8 M urea (35). In the absence of *Arabidopsis* RNA, specific products were not observed with either assay (lane 5 in *B* and lane 3 in *C*). ^{32}P-labeled DNA fragments of pBR322 cleaved with *Bst*NI were included as size standards (lane 7 in *B* and lanes 1 and 2 in *C*). (*D*) The *Arabidopsis HMG1* transcription unit is shown diagramatically. Thick lines designate untranslated leader and trailer segments, whereas the boxes indicate exons. The nucleotide sequence spanning the transcription initiation site is shown at the top of the panel. Bold residues represent the start sites determined by primer extension analysis, and underlined letters designate the positions revealed by RNase protection experiments. Nucleotides are numbered relative to the translation initiation site.

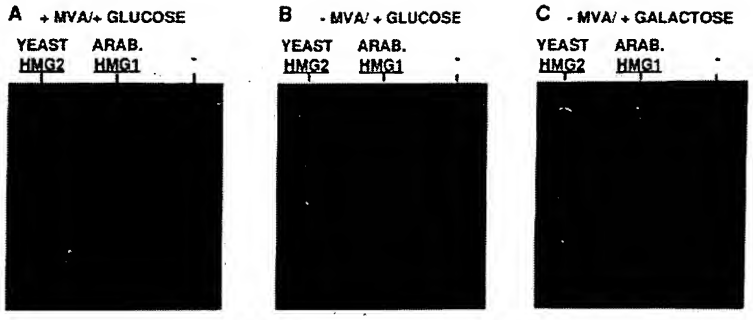

FIG. 4. Suppression of MVA auxotrophy in yeast by the *Arabidopsis* HMG-CoA reductase. A yeast strain containing disrupted copies of *HMG1* and *HMG2* (JRY1145) was transformed with a high-copy 2-μm plasmid containing either the yeast *HMG2* gene (pJR401) or the *Arabidopsis* HMG1 cDNA under yeast *GAL1* control (pYHMG1). The parent strain as well as the transformed strains were grown for 24 hr at 30°C in liquid SC medium supplemented with 2% glucose and 5 mg of mevalonic acid per ml. Serial dilutions (1:9) of each culture were prepared and then spotted onto agar plates containing the indicated nutrients. (*A*) SC medium supplemented with 30 μg of uracil and 5 mg of MVA per ml and 2% glucose. (*B*) SC medium plus 2% glucose but lacking uracil and MVA. (*C*) SC medium plus 2% galactose but lacking uracil and MVA. The most concentrated dilution of each strain contained 10^6 cells per ml, resulting in the transfer of approximately 10^4 cells (corresponding in each case to the patch of cells at the top of the figure). The amount of growth after 4 days at 30°C is shown.

Our data make the first two explanations for differential localization unlikely. Southern analysis of our strains, even at low stringency, revealed a simple pattern of hybridization consistent with the interpretation that *Arabidopsis* has a single gene for HMG-CoA reductase (data not shown). However, we cannot rule out the possibility that other less conserved genes went undetected in our experiments. There is a precedent for multiple HMG-CoA reductase genes. For example, there are two HMG-CoA reductase genes in the yeast *Saccharomyces cerevisiae*; however, these genes are sufficiently similar to cross-hybridize at high stringency (11). Production of multiple forms of HMG-CoA reductase by alternative splicing or multiple transcription initiation sites is also unlikely because both Northern analysis and S1 nuclease analysis reveal only a single species of HMG-CoA reductase transcript. Although there appears to be some microheterogeneity at the 5′ end of the message, the small differences in the expected transcripts would not lead to different translation products. Our data do not bear on the possibility that multiple forms of the protein are produced by posttranslational processing. However, antibodies produced against the HMG-CoA reductase encoded by our clone should help to test this possibility.

Note. During the period of this research, the authors became aware that A. Boronat and coworkers had simultaneously and independently cloned and characterized the gene for HMG-CoA reductase from *Arabidopsis thaliana*.

We gratefully acknowledge the generosity of Michael Basson and Jasper Rine for providing us with the yeast clone, mutant strains, and information prior to publication and for constructive discussions. Special thanks are extended to John Teem for his assistance in the design and execution of the yeast experiments. In addition, we are grateful to Elliot Meyerowitz, Nigel Crawford, and Ron Davis for *Arabidopsis* libraries. Critical reading of this manuscript by Don Rio, Steve Smale, John Teem, and Peter McCourt is especially appreciated. We also thank the members of our lab for many helpful and stimulating discussions. G.R.F. is an American Cancer Society Professor of Genetics. This research was supported by grants from the National Science Foundation (to G.R.F., DCB 8416894; and to R.M.L., Postdoctoral Fellow in Plant Biology).

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Gene, 148 (1994) 293–297

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Short Communications

Isolation and characterization of a cDNA encoding *Arabidopsis thaliana* mevalonate kinase by genetic complementation in yeast

(*erg12*-1 yeast mutant; *Saccharomyces cerevisiae*)

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SUMMARY

A 1.64-kb cDNA encoding an *Arabidopsis thaliana* mevalonate kinase (MK) was cloned by complementation of the *erg12*-1 mutation affecting MK in the yeast *Saccharomyces cerevisiae*, and the nucleotide sequence was determined. The longest open reading frame encodes a protein of 378 amino acids (aa) with a predicted molecular mass of 40 650 Da. A striking feature of the cDNA sequence is a long 5′ untranslated region (322 bp). The deduced aa sequence reveals that the plant enzyme shows strong similarities to the yeast and mammalian enzymes, especially the strong hydrophobicity percentage and several conserved regions. Southern analysis suggests that probably only one locus exists in the *A. thaliana* genome.

INTRODUCTION

In eukaryotic cells, the mevalonate pathway produces sterols involved in membrane structure and numerous kinds of isoprenoids that are vital for diverse cellular functions: respiration, glycosylations and signal transduction (Goldstein and Brown, 1990). In plants, the isoprenoid pathway has additional branches that give rise to a number of unique products, including photosynthetic pigments, growth regulators (gibberellins and abscissic acid) and a wide variety of secondary metabolites such as aromatic terpenoids and phytoalexins.

MK (ATP-mevalonate-phosphotransferase; EC 2.7.1.36), an enzyme that follows HMG-CoA reductase in the pathway, catalyzes the first phosphorylation step of MVA. Both HMG-CoA reductase and MK activities respond to external stimuli (e.g., light) in plants (Britton, 1976) and they are found both in the chloroplasts and in the cytosol (Cooke, 1977; Learned and Fink, 1989).

In vitro experiments (Dorsey and Porter, 1968; Oulmouden and Karst, 1991) have shown that MK activity is inhibited by geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) that bind competitively at the ATP-binding site on the enzyme. FPP is an important precursor at a branch point in the pathway; it is the substrate of squalene synthase, the first specific enzyme of the sterol branch, as well as of prenyl transferases involved in isoprenoid biosynthesis. These results strongly suggest that MK can play an important regulatory role

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Abbreviations: *A.*, *Arabidopsis*; aa, amino acid(s); bp, base pair(s); *erg12*-1, mutant gene encoding MK; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; kb, kilobase(s) or 1000 bp; MK, mevalonate kinase; *MK*, gene encoding MK; MVA, mevalonic acid; MVAP, MVA phosphate; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; *PGK*, gene encoding phosphoglycerate kinase; *S.*, *Saccharomyces*; *TRP1*, gene encoding *N*-(5′-phosphoribosyl) anthranilate isomerase; *URA3*, gene encoding orotidine-5′-monophosphate decarboxylase; wt, wild type; [], denotes plasmid-carrier state; ::, novel junction (fusion or insertion).

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in vivo, maintaining the size of one or more intracellular pools of FPP.

While numerous data concerning regulation of sterol biosynthetic pathway have been accumulated for mammals and yeast, little is known about plants. As a first step, we decided to clone the *A. thaliana* cDNA encoding the MK. The isolation of plant cDNAs encoding enzymes of the isoprenoid pathway is facilitated by the availability of appropriate yeast mutant strains (Karst and Lacroute, 1974; 1977) and yeast expression vectors.

EXPERIMENTAL AND DISCUSSION

(a) Isolation of an *A. thaliana* cDNA encoding MK

Temperature sensitive mutant strain AO123 (*MATα*, *erg12*-1, *ura3*-1) defective in MK (Oulmouden and Karst, 1990) was transformed by an *A. thaliana* cDNA library constructed in the yeast expression vector pFL61 (Minet et al., 1992). In this vector, the cDNAs are under the control of the strong *PGK* promoter.

Transformation experiments were performed by the lithium acetate procedure (Gietz et al., 1992). The frequency of transformation was about 3000 clones per μg DNA. Ura^+ transformants were first selected on minimal medium supplemented with ergosterol and casein hydrolysate (5 mg/ml). A second selection was made by replicating these colonies at 36°C. Among 700000 Ura^+ transformants, 30 thermoresistant clones were obtained; in only one, CR124, was thermoresistance linked to the presence of a plasmid. Plasmid pCRA was isolated from this clone and a restriction map of the insert was determined (Fig. 1).

(b) Ergosterol level and MK activity in CR124 and CR62 (*erg12*::*TRP1* [pCRA])

In order to characterize further the isolated plant cDNA, we checked whether it was able to complement not only the defective *erg12*-1 mutation in AO123, but also a disrupted *erg12* gene copy. Since *MK* is an essential gene in yeast (Oulmouden and Karst, 1990), haploid strain CR62 carrying a disrupted gene copy was obtained by sporulation of the heterozygous diploid CR41 transformed by pCRA (Table I). Like the strain CR124, strain CR62 presented a wt phenotype whatever the growth conditions tested.

The crude ergosterol content of the MK-defective strains transformed by pCRA was determined. In contrast to mutant strain AO123 defective in MK, the ergosterol level of transformed strains is quite similar to that of the wt strains FL100 and W303-1B (Table I).

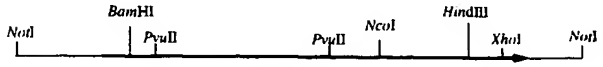

Fig. 1. Restriction map of cDNA encoding *A. thaliana* MK. The *Not*I fragment (1.64 kb) corresponds to the entire cDNA. The arrow indicates the direction and extent of the ORF.

TABLE I

Ergosterol level and MK activity in FL100, W303-1B, CR124 and CR62 strains

Strains[a]	% of 5,7-dienols[b]	MK specific activity[c]
FL100	0.77 ± 0.04 (3)	27[d]
W303-1B	0.62 ± 0.02 (3)	NT
AO123	0.20 ± 0.02 (3)	ND[e]
CR124	0.53 ± 0.04 (5)	0.37[e]
CR62	0.61 ± 0.01 (3)	0.31[e]

[a] Yeast strains: FL100 (*MATa*) and W303-1B (*MATa*, *ade2*-1, *his3*-11, *trp1*-1, *ura3*-1, *leu2*-3, *Can1*-180) are the parent strains. AO123 (*erg12*-1, *ura3*-1) and CR124 (*erg12*-1, *ura3*-1 [pCRA]) are derived from FL100. CR62 is an haploid strain of genotype (*erg12*::*TRP1*, *ade2*-1, *his3*-11, *ura3*-1, *trp1*-1, *leu2*-3, *Can1*-180 [pCRA]) derived from CR41 diploid strain. CR41 was obtained by crossing W303-1A and W303-1B; disruption was introduced by the one-step method (Rothstein, 1983): a 460-bp fragment of *ERG12* ORF was deleted (*Bgl*II digestion), *TRP1* gene was inserted and the linear *Bam*HI fragment used for integrative transformation. The heterozygous diploid CR41 prototrophic for tryptophan was then transformed by pCRA and allowed to sporulate.

[b] The amount of sterols with 5–7 dienic system was determined from $A_{281.5\ nm}$ (Servouze and Karst, 1986) and expressed as % of mg cell dry weight ± S.D. (number of independent experiments).

[c] MK specific activities were determined as described by Oulmouden and Karst (1991). The cell-free extract (12000 × *g*) was prepared as previously described for acetoacetyl-CoA thiolase assay (Servouze and Karst, 1986). Incubation mixture (final volume 100 μl) contained 20 mM ATP/10 mM $MgCl_2$. Incubation was performed at 37°C and initiated by addition of the 12000 × *g* supernatant. Results are expressed as nmol [^{14}C]MVAP formed/min per mg protein. ND, not detected; NT, not tested.

[d] 1.5 mM [^{14}C]MVA (20 nCi) or

[e] 3.8 μM [^{14}C]MVA (20 nCi).

MK activity was measured in the wt FL100 and the transformed strains CR124 and CR62 bearing the *erg12*-1 mutation and an *erg12* gene disruption, respectively. In transformed strains, the MK activity was not detected using the MK standard assay (Oulmouden and Karst, 1990). Therefore, the assay was performed under modified conditions (Table I). Results show that MK activity was recovered in transformed strains, but was much lower than in the wt strain. In addition, MK activity was detected in *E. coli* bearing pCRA (results not shown). These results clearly show that a cDNA encoding the plant MK has been isolated.

(c) cDNA sequence analysis

The 1.64-kb insert was subcloned in M13mp18 or M13mp19 vectors and sequenced by the dideoxy chain-termination method (Sanger et al., 1977). Both strands were sequenced to resolve any ambiguities. The nt

sequence of *A. thaliana* MK-encoding cDNA and the flanking DNA sequences are shown in Fig. 2.

The cDNA contains a 1134-bp ORF coding for a 378-aa peptide, assuming translational start at the third initiation codon. It has a 322-bp 5′ untranslated region before the start codon ATG at nt 323. The 3′ untranslated region (180 bp) contains the putative polyadenylation signal AATAA.

(d) Analysis of the deduced aa sequence

The deduced plant MK (378 aa, 40 650 Da) is smaller than the corresponding enzymes of *S. cerevisiae* (443 aa, 48 500 Da), human (396 aa, 42 450 Da) and rat (395 aa, 41 990 Da). Sequence comparison revealed 39, 38 and 34% identity between *A. thaliana* aa sequence and human, rat, yeast enzymes, respectively. The deduced sequence of *A. thaliana* contains about 40% hydrophobic residues, as the other described MKs. The high hydrophobicity could be linked to the hydrophobic pocket binding FPP and GPP. Fig. 3 shows the three conserved regions A, B and D already described (Schafer et al., 1992). Domain B is the most hydrophobic one and could be a good candidate for the isoprenoid-binding site. We defined a new conserved region (C) localized as domains

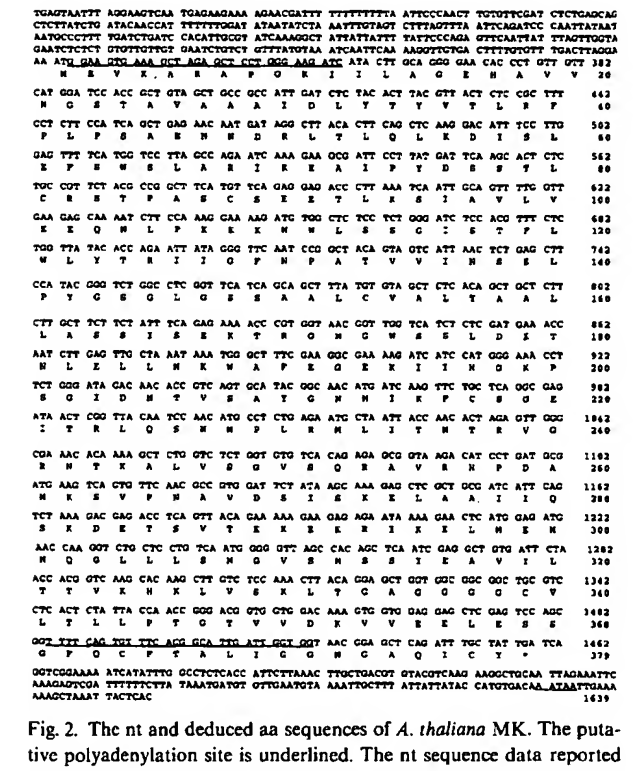

Fig. 2. The nt and deduced aa sequences of *A. thaliana* MK. The putative polyadenylation site is underlined. The nt sequence data reported appear in the EMBL under accession No. X77793. For PCR analysis, sense primer 5′-GAAGTGAAAGCTAGAGCTCCTGGGAAGATC was used with antisense primer 5′-CCACCAATCAATGCCG-TGAAACACTGAAAA. The primer sites are underlined.

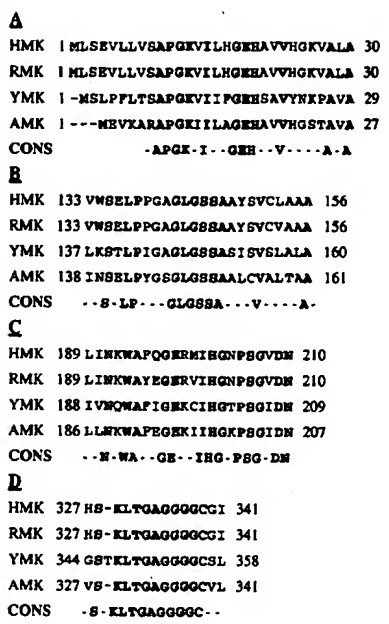

Fig. 3. The aa sequence conservation found in four regions of *A. thaliana* MK. Shown are the four regions A–D of protein sequence identity. The numbers in parentheses represent the position in the aa sequence for each MK. HMK: human (Schafer et al., 1992), RMK: rat (Tanaka et al., 1990a,b), YMK: yeast (Oulmouden and Karst, 1991), AMK: *A. thaliana*, CONS, consensus sequence.

A and B at the N terminus of the protein, in contrast to region D localized at the C terminus. Region D has been described as the ATP-binding site of kinases (Kemp and Pearson, 1990) and is strongly conserved between the four MKs.

(e) Southern and PCR analyses

To confirm that the isolated cDNA was really derived from *A. thaliana* genome, we used Southern and PCR analyses. The *A. thaliana* nuclear genome was cut with several restriction enzymes and the fragments were probed with a 0.9-kb *Hin*dIII-*Bam*HI fragment from the *A. thaliana* cDNA. The results (Fig. 4) show that only one fragment was strongly labelled. It is, therefore, very likely that the MK-encoding gene of *A. thaliana* is at only one locus. A PCR analysis was performed using the two primers shown in Fig. 2. The amplification product (1.1 kb) was electroeluted from agarose gel and sequenced (*Hin*dIII-*Bam*HI fragment, data not shown). It was identical to the cDNA sequence, proving definitively that the cDNA was derived from *A. thaliana*.

(f) Conclusions

A high number (7×10^5) of transformed clones was required to clone the *A. thaliana* cDNA encoding MK. It is likely that the difficulties were due in part to a low

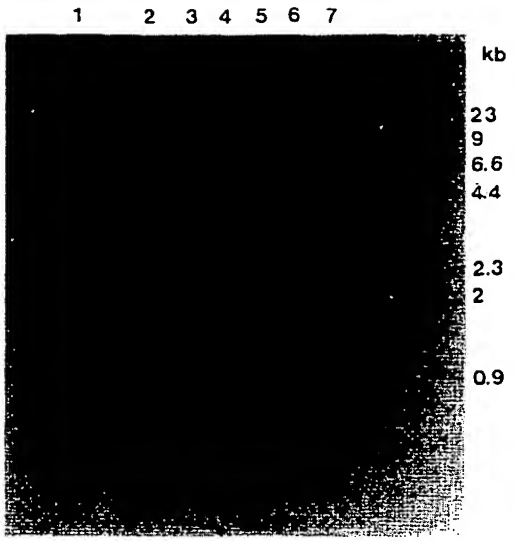

Fig. 4. Southern blot analysis of genomic DNA from *A. thaliana*. Genomic DNA was isolated from plant as described (Rogers and Bendich, 1985) and digested with different restriction endonucleases. The fragments were separated by electrophoresis and transferred to nylon membrane (Southern, 1975). The 0.9-kb *Hin*dIII-*Bam*HI fragment (Fig. 1) was electroeluted, radiolabelled by random priming and used as a probe. Hybridization and autoradiography were performed as described by Davis et al. (1986). Lane 1: 0.9-kb *Hin*dIII-*Bam*HI fragment; lanes 2–7: genomic DNA digested with *Sal*I, *Nco*I, *Kpn*I, *Hin*dIII, *Eco*RI and *Bam*HI, respectively. The molecular size standards are shown to the right.

number of copies of MK cDNA in the library, possibly related to a low expression of the *MK*.

In *S. cerevisiae*, an extremely low level of MK (Table I) is clearly sufficient to sustain a wt phenotype. The specific activity of MK in CR124 and CR62 is much lower than that of the wt strain, in spite of the fact that plant MK cDNA is under the control of the strong *PGK* yeast promoter. This low activity might be the consequence of an improper translation due to the long 5′ untranslated region (322 bp) of the cDNA (Cullin and Pompon, 1988). In addition, the 5′ sequence of plant MK cDNA contains two ATG codons and several stop codons (19) that might be implicated in the weak translation.

Since the upstream sequence contains a TATA-like box, we cloned the MK cDNA in the replicative cloning vector pFL44 (Bonneaud et al., 1992), yielding pCR12. In this construction, no promoter exists to drive the transcription of the cDNA. We could show that pCR12 was able to complement *erg12*-1 mutation and the *erg12*::TRP1 disruption. The plant cDNA can therefore be expressed in yeast without an additional promoter.

Two models have been proposed for the biosynthesis of isoprenoids in plants. The model of Rogers (1967) supposes that IPP is synthesized both in plastids and cytosol, as a precursor of carotenoids and phytosterols, respectively. In contrast, the model of Kleinig (1989) suggests that IPP is formed only in the cytosol and then is transported by a specific carrier into plastids. The Southern blot shown in Fig. 4 reveals that only one *MK* locus probably exists in the *A. thaliana* nuclear genome. In addition, the deduced aa sequence contains no putative leader peptide, strongly suggesting that the enzyme is cytosolic.

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Heterologous expression in *Saccharomyces cerevisiae* of an *Arabidopsis thaliana* cDNA encoding mevalonate diphosphate decarboxylase

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Abstract

Sequence comparison with the mevalonate diphosphate decarboxylase (MVD) amino acid sequence of *Saccharomyces cerevisiae* identified an EST clone corresponding to a cDNA that may encode *Arabidopsis thaliana* MVD (*At*MVD1). This enzyme catalyses the synthesis of isopentenyl diphosphate, the building block of sterol and isoprenoid biosynthesis, and uses mevalonate diphosphate as a substrate. Sequencing of the full-length cDNA was performed. The predicted amino acid sequence presents about 55% identity with the yeast, human and rat MVDs. The sequence of the genomic region *of A. thaliana* MVD was also obtained and Southern blot analysis on genomic DNA showed that *A. thaliana* could have at least one homologous MVD gene. In order to allow heterologous expression in *S. cerevisiae*, the MVD open reading frame (ORF) was then cloned under the control of the yeast *PMA1* strong promoter. When expressed in yeast, the *A. thaliana* cDNA complemented both the thermosensitive *MN19-34* strain deficient in MVD, and the lethal phenotype of an *ERG19* deleted strain. However, the wild-type sterol content was not fully restored suggesting that the *A. thaliana* MVD activity may not be optimal in yeast. A two-hybrid assay was also performed to evaluate homodimer formation of the *A. thaliana* MVD and heterodimer formation between the plant and yeast heterologous enzymes.

Introduction

The mevalonate pathway in eukaryotic cells leads to the synthesis of both sterols and isoprenoids. Sterols are mainly involved in plasma membrane structure and are precursors of steroid biosynthesis. Moreover, in yeast, ergosterol is implicated in the regulation of the cell cycle [17]. Isoprenoids take part in diverse cellular functions, such as respiration, protein glycosylation and signal transduction [8]. In higher plants, this pathway leads also to a large number of plant-specific molecules including photosynthetic pigments, growth regulators (e.g. gibberellins and abscisic acid), phytotoxins, phytoalexins and other plant defence compounds. A wide variety of secondary metabolites such as aromatic terpenoids and natural rubbers are also derived from this pathway [1, 5].

Mevalonate diphosphate decarboxylase (MVD; EC L-1-1-33) is a key enzyme in the isoprenoid and sterol biosynthetic pathway. It catalyses the first step of isoprenoid biosynthesis, i.e. the decarboxylation of the six-carbon mevalonate diphosphate (MVA-PP) to the five-carbon isopentenyl diphosphate (IPP). IPP is the basic skeleton of the biosynthesis of isoprenoids. The decarboxylation catalysed by MVD is accompanied by the dehydratation of the substrate and the hydrolysis of one molecule of ATP and requires Mg^{2+} [3, 11].

In plants, sterols and isoprenoid-derived molecules are localized to various subcellular compartments in which they fulfil their physiological function: sterols

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under the accession numbers Y14325A and Y17593.

are enriched in the plasma membrane, chlorophyll is exclusively found in chloroplasts, carotenoids in chromoplasts, ubiquinone in mitochondria. The subcellular compartmentalization of the enzymes involved in the synthesis of these molecules has been extensively investigated but remains poorly documented [1, 5, 9, 12]. A novel non-mevalonate pathway for the synthesis of IPP has been discovered in bacteria [19] and green algae [22] and, recently, has also been demonstrated as being the source of xantophyla and carotenoid synthesis of higher plants [14]; these molecules are synthesized in the plastid compartment. It is of interest to check the localization of the enzymes involved in the sterol/isoprenoid biosynthetic pathway. Therefore, isolation of a plant MVD cDNA could be helpful to characterize the precise localization of this enzyme, and more generally to elucidate the regulation and the compartmentalization of the two pathways.

The cDNA encoding the MVD of budding yeast, human and rat have been recently isolated and characterized [2, 25, 26]. During these studies, several EST clones from species such as *Arabidopsis thaliana*, *Oryza sativa* and *Caenorhabditis elegans* were identified as potentially corresponding to cDNAs encoding MVD from these species. In order to get more information about the plant MVD and about the early steps of isoprenoid biosynthesis in plants, we decided to sequence the full-length cDNA encoding *A. thaliana* MVD from the largest EST clone identified and to express it in yeast under the control of an appropriate promoter. We report here on the molecular characterization of the *A. thaliana* MVD DNA (both the cDNA and the genomic coding region) and on the functional complementation of two MVD-deficient *Saccharomyces cerevisiae* strains with the *A. thaliana* enzyme.

Materials and methods

Strains and plasmids

The following *Saccharomyces cerevisiae* strains were used: the wild-type strain FL100 (ATCC 28 383, *MAT* a), the mutant strain *MN19-34* (*MAT* a, *erg19-34*, *ura3-1*, *trp1-1*) temperature-sensitive and defective for MVD activity [4], the deleted diploid strain TB1Δ (*erg19::TRP1/ERG19, ura3-1/ura3-1, trp1-1/trp1-1*) [2], and the strain L40 (*MAT* a, *trp1*, *leu2*, *his3*, *ade2*, *LYS2::lexA-HIS3*, *URA3::lexA-lacZ*).

The *Escherichia coli* strains DH5α and XL1Blue (Stratagene) were used for plasmid amplification.

The Newman EST clone 123P21T7 [16] carrying the cDNA encoding *Arabidopsis thaliana* MVD was kindly provided by Dr Keith R. Davis from the Arabidopsis Biological Resource Center of Ohio State University. This clone originates from the *A. thaliana* cDNA library built in the *Escherichia coli* DH10b strain (Promega). The cDNA contained in the EST clone 123P21T7 [16] is inserted as a *SalI/NotI* restriction fragment in the polylinker of the double-strand plasmid pZL1, derived from the vector λZipLox (Promega). pZL1 allows the α-complementation test and contains the complementary sequences for the universal and reverse primers.

The *E. coli* replicative vectors pUC18 and pBlue-Script SK+ (Pharmacia) were used for subcloning and sequencing.

The *E. coli* replicative vector pGEMT-easy (Promega) that allows the direct cloning of PCR fragments, was used to clone the genomic MVD region amplified by PCR. The resulting plasmids were called pHC127AT and pHC128AT (both orientations).

The pNEV-1 [27] and pRS316 [24] *S. cerevisiae/E. coli* shuttle vectors were used to subclone the coding region of the cDNA encoding MVD generated by PCR. pNEV-1 is a replicative plasmid in which a unique *Not*I restriction site allows the insertion of any cDNA whose transcription is under the control of the strong plasma membrane ATPase 1 (*PMA1*) promoter from *S. cerevisiae*, thereby allowing expression in yeast. The resulting plasmid was called pHC124AT.

The 3.7 kb *Hin*dIII fragment of pHC124AT containing the *A. thaliana* ORF flanked by *PMA1* promoter and terminator was inserted into the *Hin*dIII site of the polylinker of the centromeric plasmid pRS316. The resulting plasmid was called pHC126AT.

The plasmids pBTM116 [28] (kindly provided by Dr Jacques Camonis, INSERM U-248, Paris) containing the *lexA*-DNA binding domain and pGAD424 (Clontech) containing the *GAL4* activation domain were used for two-hybrid assay. The *A. thaliana* cDNA amplified by PCR was cloned into the *Eco*RI/*Bam*HI sites of pBTM116 and pGAD424, resulting in pLEXA-AtMVD and pGAD-AtMVD.

Preparation of plant genomic DNA

Genomic DNA was prepared from *A. thaliana* young plants (2-week old, prior to flowering) grown on solid Murashige & Skoog medium (Ducheta), according to the Rogers and Bendich method [18], using 500 mg of

frozen plants (all tissues). Tobacco genomic DNA was kindly provided by Dr Rémi Lemoine.

Preparation of yeast total RNA

15 OD_{600} units of exponentially growing yeast cells were pelleted and resuspended in RNA extraction buffer (100 mM LiCl, 1 mM EDTA, 100 mM Tris-HCl pH 7.5). A mix of 0.5 ml PCI (phenol/chloroform/isoamyl alcohol 25:24:1 v/v/v), 0.5 ml RNA extraction buffer, 10 μl 10% SDS and 0.5 ml of glass beads was added to the cells. Eight cycles of 30 s vortexing / 30 s incubation on ice were performed, and the aqueous phase was re-extracted twice with one volume of PCI. Aqueous phase was then adjusted with 0.3 M sodium acetate and RNA were precipitated with 2.5 volumes of ethanol and by storing at least 1 h at −20 °C. The RNA pellet was then resuspended in water treated with DEPC (diethyl pyrocarbonate) 1:1000 v/v.

Preparation of plant total RNA

500 mg of frozen young *A. thaliana* plants (2 weeks old, all tissues) grown on solid Murashige & Skoog medium (Ducheta) were ground with liquid nitrogen, and the powder obtained was resuspended in 600 μl of REB buffer (25 mM Tris-HCl pH 8, 25 mM EDTA pH 8, 75 mM NaCl, 1% SDS, 1 M 2-mercaptoethanol). Two extractions with one volume of PCI were performed, and the aqueous phase was washed with one volume of CI (chloroform/isoamyl alcohol 24:1 v/v). LiCl was added to a final concentration of 2 M, and precipitation was performed overnight at 4 °C. The RNA pellet was then washed with 2 M LiCl and resuspended in DEPC water. RNA was precipitated with 1:10 volume of 3 M sodium acetate pH 5.2 and 2 volumes of ethanol for 20 min at −20 °C. The RNA pellet was washed twice with 70% ethanol and resuspended in DEPC-treated water.

Polymerase chain reaction

The oligonucleotides MVD3 (5′-AAAGCGGCCGC ATGGCGGAGGAGAAATGG-3′) and MVD4 (5′-AAAGCGGCCGCTTATTTGGGGAGGCCAGT-3′) were used to amplify the complete open reading frame encoding *A. thaliana* MVD. The PCR product has a size of 1258 bp. These primers were designed according to the sequence of the whole cDNA encoding *A. thaliana* MVD. The sequence of MVD3 corresponds to the 5′ part of the cDNA and starts exactly at the ATG codon. The sequence of MVD4 corresponds to the complementary sequence of the 3′ part and ends exactly at the stop codon (TAA). Both MVD3 and MVD4 are flanked by a *Not*I linker, allowing the subcloning of the coding region in the *Not*I site of pNEV-1 cloning site. The resulting construct was called pHC124AT.

The oligonucleotides MVD5 (5′-ATGGCGGAGG AGAAATGGGTG-3′) and MVD6 (5′-TTATTTGGGG AGGCCAGTTTG-3′) were used to amplify the genomic MVD coding region, from the start codon ATG to the stop codon TAA. MVD5 starts exactly at the ATG, and MVD6 ends exactly at the TAA. No linkers were added. The 2837 bp PCR product was directly cloned in pGEM-T Easy (Promega), resulting in the pHC127AT and pHC128AT constructs (both orientations).

The oligonucleotides MVD10 (5′-AAAGAATTC ATGGCGGAGGAGAAATGGG-3′) flanked by an *Eco*RI linker and MVD11 (5′-AAAGGATCCTTATTT GGGGAGGCCAGTTTG-3′) flanked by a *Bam*HI linker, were used to amplify the *A. thaliana* MVD coding region from start to stop codons. The fragment obtained was controlled by sequencing and fused in frame with the *lexA*-DNA binding domain of pBTM116 [28] and to the *GAL4*-activation domain of pGAD424 (Clontech), resulting in plasmids pLEXA-AtMVD and pGAD-AtMVD used for the two-hybrid assay.

The polymerase chain reaction was performed with Pharmacia r-Taq DNA polymerase, under the conditions described by the supplier. The temperature of annealing was 52 °C, and 30 cycles were performed.

Sequence analysis

The clone 123P21T7 is available in the EMBL data library under the accession number of gb|T44736|T447 36 7999. This clone was identified by homology search against the yeast MVD cDNA sequence, using the Experimental GENINFO (R) BLAST Network Service (NCBI).

The EST clone 123P21T7 kindly provided by Dr Keith Davis was directly sequenced using universal and reverse primers. Several deletions and subcloning were performed to obtain the complete cDNA sequence on both strands.

The pHC127AT and pHC128AT plasmids containing the genomic region in both orientations, and several subclones, were sequenced using the universal and reverse primers, allowing the obtention of the

whole genomic region sequence, from the start codon to the stop codon.

The sequences were obtained with the dideoxynucleotide method [21], using the Autoread Sequencing kit with the ALF DNA sequencer (Pharmacia), and the ABI PRISM Dye Terminator Cycle Sequencing kit with the ABI PRISM A310 sequencer (Perkin Elmer). Sequences were assembled and analysed with the DNASIS program (Hitachi). The GCG program PILEUP was used to align the translated sequence with amino acid sequences from the SwissProt data bank; the accession numbers are U49260 (human MVD), U49261 (yeast MVD) and U53706 (rat MVD).

Southern blot analysis

10 μg of *A. thaliana* and *Nicotiana tabacum* genomic DNA were digested with either *Bgl*II or *Eco*RI. The DNA fragments were separated in a 0.8% agarose gel, blotted onto a nylon membrane (Hybond N+, Amersham) and hybridized with a ^{32}P-dCTP-labelled probe. The 752 bp *Kpn*I/*Pst*I fragment from MVD cDNA was radiolabelled by random priming. Hybridization was performed at 65 °C in Quick Hybrid Buffer (Amersham). The washes of the membrane were performed under high stringency conditions (2×5 min in 2×SSC, 0.5% SDS at room temperature, 20 min in 1×SSC, 0.5% SDS at 68 °C, 3×20 min in 0.1×SSC, 0.5% SDS at 68 °C).

Northern blot analysis

10 μg and 20 μg of total RNA were separated in a 1.2% denaturing agarose gel containing 12.32 M formaldehyde, and blotted onto a nylon membrane (Hybond N, Amersham). Hybridization was performed at 65 °C in a buffer composed of 0.25 M sodium phosphate pH 7.2, 6.6% SDS, 1 mM EDTA and 1% v/w BSA, with the 752 bp *Kpn*I/*Pst*I probe described above. Washes were performed as follows: 2×5 min in 2×SSC, 0.5% SDS at room temperature, 20 min in 1×SSC, 0.5% SDS at 68 °C and 2×20 min in 0.1×SSC, 0.5% SDS at 68 °C.

Reverse transcription and PCR reactions

The first strand of the cDNA synthesis was performed using the Superscript II Reverse Transcriptase (Gibco-BRL) from 5 μg of total RNA. The synthesis of the second strand was performed by PCR with *Taq* DNA polymerase (Gibco-BRL), using the two *A. thaliana* MVD-specific primers MVD5 and MVD6, with an annealing temperature of 52 °C. The reverse transcription and PCR were performed according to the conditions described by the supplier.

Yeast functional complementation assays

Plasmid pHC124AT, containing the coding region of *A. thaliana* MVD, was used to transform the yeast mutant strain *MN19-34* using the lithium acetate method described by Gietz *et al.* [7]. Transformed cells (HC0 strain) were spotted on complete medium (YPD) at 28 °C and 36 °C to analyse the functional complementation of the plant cDNA.

The heterozygous deleted diploid strain TB1Δ was transformed with either pHC124AT or pHC126AT, using the same method, and transformed cells were forced to sporulate to obtain viable transformed haploid cells. The remaining diploid cells were then killed by diethyl ether. Haploid transformed cells bearing both the disrupted allele and the plasmid-borne MVD (HC1: *erg19::TRP1*, *ura3-1*, *trp1-1* [pHC124AT] and HC2: *erg19::TRP1*, *ura3-1*, *trp1-1* [pHC126AT]) were selected on minimal medium (YNB). Haploidy of the prototrophic strains was verified by crosses with appropriate haploid strains of known mating type. Plasmid loss was analysed on a 5-fluoro-orotic acid (5-FOA) containing medium prepared as described by Guthrie *et al.* [10].

Sterol level measurement

Sterols were extracted from yeast as described previously by Marcireau *et al.* [15]. Yeast strains were grown for 16 h in complete medium (YPD). The amount of sterol with conjugated 5–7 double bonds (75% ergosterol and 25% ergosta-5,7-dienol) was determined from the maximum absorbance at 281.5 nm [23], and expressed as % of cell dry weight: % 5,7 = $OD_{281.5nm}$ × volume of heptanolic phase (ml) × 396 / 11500 × dry weight (mg). The sterol composition of each extract was then analysed by gas chromatography, with a Carlo Erba GC 6000 chromatograph, on a SE 30 column. Cholesterol (Sigma) was used as the internal standard to determine relative retention times (in min) as described by Bergès *et al.* [2].

Two-hybrid assay

The recipient strain L40 was co-transformed with both plasmids pLEXA-AtMVD and pGAD-AtMVD, using the lithium acetate method [7]. The resulting

strain was called T1. Strain T2 was obtained by co-transformation of strain L40 with pLEXA-AtMVD and pGAD-19wt containing the *S. cerevisiae* gene *ERG19* encoding MVD, fused in frame with the *GAL4*-activation domain of pGAD424. Strain T3 was obtained by co-transformation of L40 with pLEXA-19ts containing the *erg19-34* allele from the yeast temperature-sensitive strain *MN19-34*, fused in frame with the *lexA*-DNA-binding domain of pBTM116, and pGAD-AtMVD. Strain T4 was obtained by co-transformation of L40 with pLEXA-AtMVD and pGAD-19ts containing the *erg19-34* allele fused in frame with *GAL4*-activation domain (Cordier *et al.*, manuscript in preparation). Co-transformed clones were selected on minimium medium YNB supplemented with adenine and histidine (50 μg/ml). Independent clones were grown on minimum medium YNB supplemented with adenine (50 μg/ml) for the detection of the activation of the *HIS3* reporter gene.

Independent transformants were grown overnight on a Whatman #40 filter on complete YPD medium. Filters were immersed 10 min in liquid nitrogen and then placed on top of a Whatman #3 filter prewet in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 38 mM 2-mercaptoethanol, pH 7), containing 0.2 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal). XGal assays were performed by incubating the filters for 3 h at 37 °C. Blue coloration after incubation is indicative of β-galactosidase activity.

The transformants were grown to stationary phase in minimum medium containing 3% glucose (w/v) supplemented with adenine and histidine (50 μg/ml). The β-galactosidase activity was determined from crude protein extracts as described previously [20].

Results

Identification of an A. thaliana *cDNA encoding MVD*

Database searches with the amino acid sequence of the yeast mevalonate diphosphate decarboxylase led to the identification of an EST that may contain the cDNA encoding the *A. thaliana* homologue of this enzyme [25]. The partial nucleotide sequence available in the data library was 471 bp long, and its deduced amino acid sequence presented 56.7% identity over 74 amino acids with the *S. cerevisiae* MVD amino acid sequence. Since this sequence was homologous to the N-terminal part of the yeast MVD, the 123P21T7 EST clone was expected to contain the complete ORF. This clone was provided by Dr Keith R. Davis from the Arabidopsis Biological Resource Center of the Ohio State University for further analysis.

Analysis of the cDNA and the deduced amino acid sequence

We first established a restriction map of the *NotI/SalI* insert contained in 123P21T7 by digestion with several restriction enzymes (Figure 1). The estimated size of the *NotI/SalI* insert was 1800 bp. A nucleotide sequence of 1695 bp was obtained, containing a 1239 bp long open reading frame (*At*MVD1 cDNA, EMBL accession number Y14325A). The 3′ end of the insert including the poly(A) region was not sequenced. The ATG start codon is located at position 118 relative to the first nucleotide of the insert, and the TAA stop codon at position 1354. The open reading frame potentially encodes a protein of 412 residues with a predicted molecular mass of 45 585 Da and a theorical isoelectric point of 6.33. A putative polyadenylation signal AATAAA is found at position 1634 in the 3′-untranslated region.

The predicted amino acids sequence of *A. thaliana* MVD was aligned with the various MVD sequences available: rat MVD (accession number U53706), human MVD (U49260) and *S. cerevisiae* MVD (U49261), and the results are summarized in Figure 2. The protein presents 57%, 56% and 54% strict identity, and 69%, 72% and 71% similarity with yeast, human and rat MVDs, respectively. The *A. thaliana* MVD is clearly homologous to the other eukaryotic MVDs previously described, especially in its N-terminus half.

Analysis of the A. thaliana *MVD genomic region*

The genomic region corresponding to the MVD coding region was obtained by PCR amplification from *A. thaliana* genomic DNA, using the primers MVD5 and MVD6 that were designed to target the region located between the start codon and the stop codon. The entire sequence of the 2837 bp genomic region that was isolated was subsequently identified (*At*MVD1 gene, EMBL accession number Y17593). Nine exons and eight introns were identified by sequence comparison with the cDNA. The localization and sequence of the 5′ and 3′ intron splice sites are reported in Table 1. The splice sites were compared to the *Arabidopsis* intron splice-site table

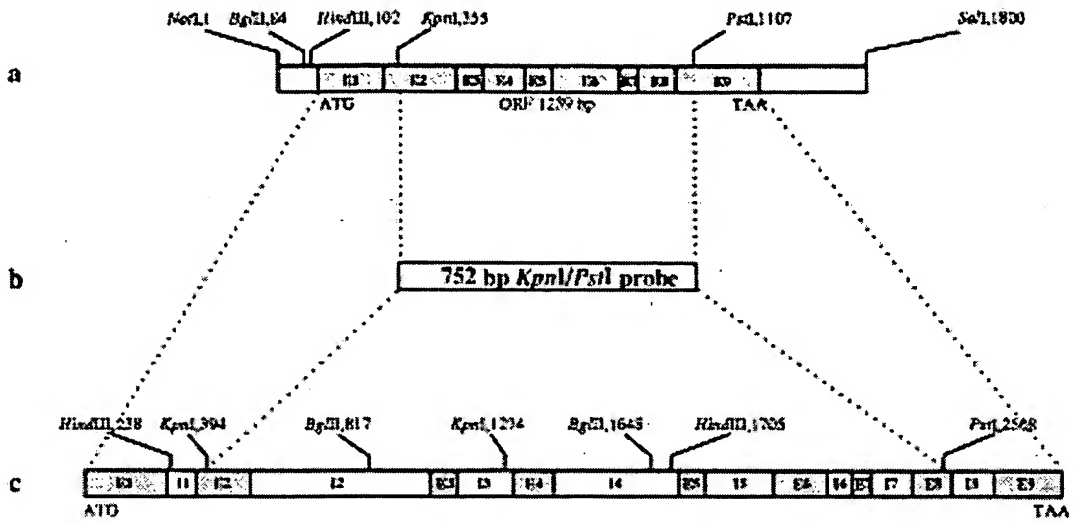

Figure 1. Organization of *Arabidopsis thaliana* MVD cDNA and genomic region, and Southern blot analysis. a. Map of the whole 1800 bp MVD cDNA containing a 1239 bp ORF. b. *KpnI*/*PstI* (752 bp) probe used for Southern analysis. It overlaps a 2114 bp region on the genomic DNA. c. Schematic drawing of the genomic region. The region from the start to the stop codon is 2837 bp long and contains nine exons (E1 to E9) and eight introns (I1 to I8). d. Southern blot analysis under high-stringency conditions. Lane 1, *A. thaliana* genomic DNA digested with *Bgl*II (10 μg); lane 2, *A. thaliana* genomic DNA digested with *Eco*RI (10 μg); lane 3, tobacco genomic DNA digested with *Bgl*II (10 μg); lane 4, tobacco genomic DNA, digested with *Eco*RI (10 μg).

```
                1                                                    50
Human           MASEKPLA.A VTCTAPVNIA VIKYWGKRDE ELVLPINSSL SVTLHQDQLK
Norway rat      MASEKPQDLM VTCTAPVNIA VIKYWGKRDE ALILPINPSL SVTLHQDQLK
A. thaliana     MAEEK.WVVM VTAQTPTNIA VIKYWGKRDE VRILPINDSI SVTLDPDHLC
Yeast           .....MTVYT ASVTAPVNIA TLKYWGKRDT KLNLPTNSSI SVTLSQDDLR
Consensus       ---------- -----P-NIA --KYWGKRD- ---LP-N-S- SVTL--D-L-

                51                                                   100
Human           TTTTAVISKD FTEDRIWLNG REEDVGQPRL QACLREIRCL ARKRRNSRDG
Norway rat      TTTTAAISKD FTEDRIWLNG REEDVGQPRL QACLREIRRL ARKRRSTGDG
A. thaliana     TLTTVAVSPS FDRDRMWLNG KEISLSGSRY QNCLREIRSR ADDVEDKEKG
Yeast           TLTSAATAPE FERDTLWLNG EPHSIDNERT QNCLRDLRQL RKEMESKDAS
Consensus       T-T------- F--D--WLNG --------R- Q-CLR--R-- ----------

                101                                                  150
Human           DPLPSSLSCK ..VHVASVNN FPTAAGLASS AAGYACLAYT LARVYGVESD
Norway rat      DALPLSLGYK ..VHVASVNN FPTAAGLASS AAGYACLAYT LARVYGVEGD
A. thaliana     IKIAKKDWEK LHLHIASHNN FPTAAGLASS AAGFACLVFA LAKLMNVNED
Yeast           LPTLS....Q WKLHIVSENN FPTAAGLASS AAGFAALVSA IAKLYQLPQS
Consensus       ---------- ---H--S-NN FPTAAGLASS AAG-A-L--- -A--------

                151                                                  200
Human           ...LSEVARR GSGSACRSLY GGFVEWQMGE QADGKDSIAR QVAPESHWPE
Norway rat      ...LSEVARR GSGSACRSLY GGFVEWQMGE QADGKDSIAR QIAPEWHWPQ
A. thaliana     PSQLSAIARQ GSGSACRSLF GGFVKWNMGN KEDGSDSVAV QLVDDKHWDD
Yeast           TSEISRIARK GSGSACRSLF GGYVAWEMGK AEDGHDSMAV QIADSSDWPQ
Consensus       ----S--AR- GSGSACRSL- GG-V-W-MG- --DG-DS-A- Q------W--

                201                                                  250
Human           LRVLILVVSA EKKLTGSTVG MRASVETSPL LRFRAESVVP ARMAEMARCI
Norway rat      LRVLILVVSA EKKPTGSTVG MQTSVATSTL LKFRAESIVP ERMKEMTRCI
A. thaliana     LVIIIAVVSS RQKETSSTSG MRESVETSLL LQHRAKEVVP VRILQMEEAI
Yeast           MKACVLVVSD IKKDVSSTQG MQLTVATSEL FKERIEHVVP KRFEVMRKAI
Consensus       ------VVS- --K---ST-G M---V-TS-L ---R----VP -R---M---I

                251                                                  300
Human           RERDFPSFAQ LTMKDSNQFH ATCLDTFPPI SYLNAISWRI IHLVHRFNAH
Norway rat      QEQDFQAFAQ LTMKDSNQFH ATCLDTFPPI SYLNDTSRRI IQLVHRFNAH
A. thaliana     KNRDFTSFTK LTCSDSNQFH AVCMDTSPPI FYMNDTSHRI ISLVEKWNRS
Yeast           VEKDFATFAK ETMMDSNSFH ATCLDSFPPI FYMNDTSKRI ISWCHTINQF
Consensus       ---DF--F-- -T--DSN-FH A-C-D--PPI -Y-N--S-RI I------N--

                301                                                  350
Human           HGDTKVAYTF DAGPNAVIFT LDDTVA.EFV AAVWHGFPPG SN........
Norway rat      HGQTKVAYTF DAGPNAVIFT LEDTVA.EFV AAVRHSFPPA AN........
A. thaliana     AGTPEIAYTF DAGPNAVMIA RNRKVAVELL QGLLYCFPPK PDTDMKSYVL
Yeast           YGETIVAYTF DAGPNAVLYY LAEN.ESKLF AFIYKLFGSV PGWDKKFTTE
Consensus       -G----AYTF DAGPNAV--- ---------- ------F--- ----------

                351                                                  400
Human           GDTFLKGLQV RPAPLSAELQ AALAMEPTPG GVKYIIVTQV GPGPQILDDP
Norway rat      GDKFLKGLQV APVLLSDELK TSLATEPSPG GVQYIIATQV GPGPQVLDDP
A. thaliana     GDTSIVKEAG LEGELPQGIK DKIGSQDQKG EVSYFICSRP GRGPVVLQDQ
Yeast           QLEAFNHQFE SSNFTARELD LELQKD.... .VARVILTQV GSGP...QET
Consensus       ---------- ---------- ---------- -V---I---- G-GP------

                401          416
Human           CAHLLGPD.G LPKPAA
Norway rat      HMHLLGPD.G LPQRDL
A. thaliana     TQALLHPQTG LPK...
Yeast           NESLIDAKTG LPKE..
Consensus       ---L-----G LP----
```

Figure 2. Alignment of the amino acid sequence of *Arabidopsis thaliana* mevalonate diphosphate decarboxylase with the proteins encoded by rat, human and yeast genes. The alignment was performed using the PILEUP GCG program. The accession numbers of *A. thaliana*, rat, human and yeast MVDs are Y14325A, U53706, U49260 and U49261 respectively.

Table 1. Organization of the genomic region of *A. thaliana* MVD. The genomic part corresponding to the translated region is composed of nine exons and eight introns. The positions were determined from the predicted ATG start codon. 5′ and 3′ intron splice-sites are indicated for each intron.

Exons	Size (bp)	Position	Introns	Size (bp)	Position	5′ splice site	3′ splice site
E1	210	1 – 210	I1	156	211 – 366	AAG ! GTA	TAG ! GAA
E2	199	367 – 565	I2	455	566 – 1020	TAG ! GTA	CAG ! TTT
E3	64	1021 – 1084	I3	232	1085 – 1316	AAG ! GTA	TAG ! GCA
E4	145	1317 – 1461	I4	309	1462 – 1770	GTG ! GTA	CAG ! GTT
E5	87	1771 – 1857	I5	155	1858 – 2012	AAG ! GTA	TAG ! GAA
E6	158	2013 – 2170	I6	99	2171 – 2269	CAG ! GTA	CAG ! GAT
E7	49	2270 – 2318	I7	112	2319 – 2430	GAG ! GTA	CAG ! ATT
E8	125	2431 – 2555	I8	80	2556 – 2635	GAG ! GTG	TAG ! TTA
E9	202	2636 – 2837					

available in the *A. thaliana* nucleotide sequence database (http://genome-www.stanford.edu/Arabidopsis) and were found to match perfectly with the consensus sequences. A map of the organization of the exons and introns is presented in Figure 1.

Southern blot analysis

The 752 bp *KpnI/PstI* probe prepared from the *A. thaliana* MVD cDNA overlaps a 2114 bp region on the genomic part including exons 2 to 8 and introns 2 to 7 (Figure 1). The genomic DNA was digested with two restriction enzymes, *BglII* or *Eco*RI. It is important to notice that the washing steps were done under high-stringency conditions (Materials and methods).

Three *BglII* fragments were expected to hybridize the probe, since there are two *BglII* sites in the region covered by this probe. The size of one of these fragments should be 831 bp. The size of the two other fragments could not be determined, since the untranslated regions upstream from the start codon and downstream from the stop codon have not been isolated.

Only one fragment was expected to be detected for the *Eco*RI digestion, since there is no *Eco*RI site in the region overlapped by the probe. The size of this *Eco*RI fragment should be higher than 2837 bp, but could not be precisely determined for the same reasons.

The results of the Southern analysis are presented in Figure 1. Four *BglII* fragments hybridized to the probe, with estimated sizes of 2700 bp, 1250 bp, 850 bp and 760 bp respectively, and two *Eco*RI fragments, which estimated sizes were 8400 bp and 4050 bp. The number of bands obtained in comparison to the number of expected fragments for the *At*MVD1 gene suggests the probable existence of a second gene. Given the high-stringency conditions used for the washing steps (see Materials and methods), the degree of homology between the two genes should be high.

On the other hand, no hybridization could be detected on tobacco genomic DNA (Figure 1), even when lower-stringency conditions were used (not shown). This may be due to the larger size of the tobacco genome relative to *A. thaliana*.

Analysis of the MVD expression in A. thaliana

Expression of MVD in the plant was studied by northern blot, with the same MVD-specific probe as for the Southern analysis. Two different amounts (10 and 20 μg respectively) of total RNA extracted from whole young plants were loaded. The results are shown in Figure 3, and reveal the presence of a very faint band of the expected size. The signal could only be detected using 20 μg of total RNA. This result could be explained by a low expression of MVD mRNA in the whole plant, or a tissue-specific expression.

In order to increase the detection level of the MVD RNA, a reverse transcription reaction followed by PCR with *A. thaliana* MVD-specific primers MVD5 and MVD6 (Materials and methods) was performed on *A. thaliana* total RNA. The results presented in Figure 3 show that a single specific fragment of the expected size (1240 bp) was amplified, confirming the northern blot result.

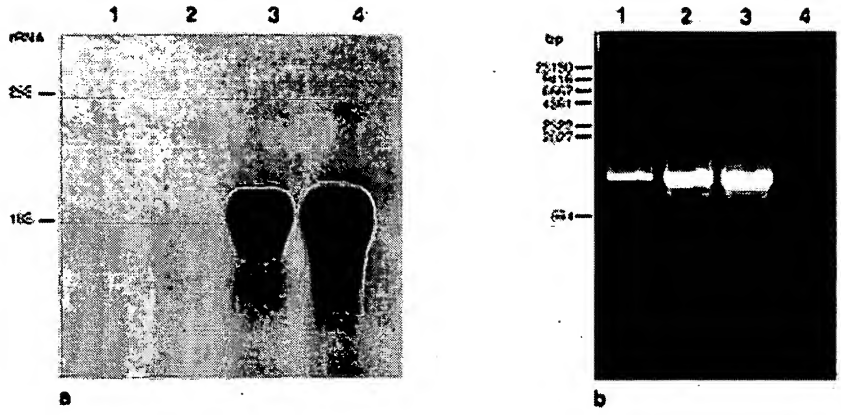

Figure 3. Expression of *Arabidopsis thaliana* MVD in plant and heterologous expression in yeast. a. Expression of *Arabidopsis thaliana* MVD studied by northern blot analysis, using the 752 bp *KpnI/PstI* MVD-specific probe and hybridized to 10 μg (lane 1) and 20 μg (lane 2) of *A. thaliana* RNA prepared from whole 2-week old plants (Materials and methods), and 10 μg (lane 3) and 20 μg (lane 4) of total RNA of a yeast strain deleted for MVD and bearing a single copy of the plant MVD cDNA (HC2: *erg*19::*TRP*1 [pHC126AT]). b. Reverse-transcription PCR (RT-PCR) with *A. thaliana* MVD-specific primers (MVD5 and MVD6). The reaction was performed on 5 μg of plant total RNA (lane 1) and 1 μg of yeast total RNA (HC2, lane 2). 123P21T7 DNA was added as a positive control and size indicator (lane 3); a negative control without DNA was also included (lane 4).

Heterologous expression in yeast of the plant cDNA

In order to further characterize the putative *A. thaliana* MVD, we decided to check whether the plant enzyme is functional in yeast, by assaying its ability to complement two yeast strains deficient in MVD. One of these two strains is the temperature-sensitive strain *MN19-34* [4]. It harbours a recessive mutation that replaces Leu-79 by a Pro in the coding sequence of the *ERG19* gene [2]. Due to the leaky character of the defective allele, when the strain is grown at permissive temperature, the sterol biosynthetic pathway produces the minimal ergosterol amount required for growth (ca. 0.2% of dry weight) and no intermediates of the pathway can be detected ([2], and Table 2). The second strain bears a deleted copy of the *ERG19* gene, and therefore cannot survive without any plasmid-borne MVD [2].

We first constructed the pHC124AT plasmid containing the whole coding region of *A. thaliana* MVD, including its own start and stop codons, cloned under the control of the promoter and terminator of the yeast plasma membrane ATPase gene (*PMA1*) [27]. The *PMA1* promoter is a strong promoter and, moreover, the vector used is a high-copy-number plasmid. Thus, this construction should lead to the overproduction of the mRNA encoding the *A. thaliana* MVD. To achieve this construct, the coding sequence of the *A. thaliana* MVD was amplified by polymerase chain reaction (PCR) from 123P21T7, using the MVD3 and MVD4 oligonucleotides that were designed according to the cDNA sequence, and cloned into the pNEV-1 vector to generate the pHC124AT plasmid. The sequence of the amplified product was verified and remains identical to the cDNA sequence.

This construct was used to transform the yeast temperature-sensitive strain defective in MVD activity (*MN19-34* strain). The transformants were first selected at permissive temperature and then replica-plated at 36 °C. All the Ura^+ transformants could clearly grow at non-permissive temperature, although slower than the wild type, as shown in Figure 4, whereas the parental mutant strain (*MN19-34*) could not grow at this temperature. *MN19-34* harbouring the pHC124AT plasmid was called HC0.

In order to express the plant cDNA in a yeast strain deleted for MVD, we decided to transform with pHC124AT a yeast diploid strain in which a copy of the functional *ERG19* gene is disrupted by the *TRP*1 marker (TB1Δ strain). We then analysed the haploid progeny of these transformed cells after sporulation. Haploid strains bearing both the yeast disrupted gene copy and the plant cDNA (Ura^+, Trp^+) were obtained (HC1). Surprinsingly we found that they were indistinguishable from the wild-type strain, whatever the growth conditions (Figure 4), in contrast to HC0. No plasmid loss was observed after five days of subculturing on complete medium. Moreover, the

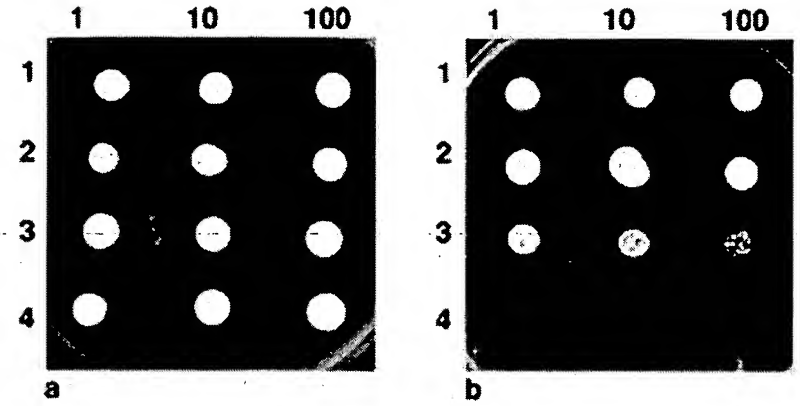

Figure 4. Yeast complementation with the plant cDNA encoding MVD. Growth of the yeast mutant strain *MN19-34* defective in MVD activity, complemented with the plant cDNA (HC0 strain: *MN19-34* [pHC124AT], lane 3) was compared to *MN19-34* transformed with the pNEV-1 plasmid (lane 4, negative control), and to the wild-type FL100 (lane 2). The growth of the haploid disrupted strain complemented with the plant cDNA (HC1: *erg*19::*TRP*1 [pHC124AT], lane 1) was also assayed. The strains were grown on complete medium at 28 °C (a) and 36 °C (b), using three dilutions of yeast cell suspensions (1, 10, 100).

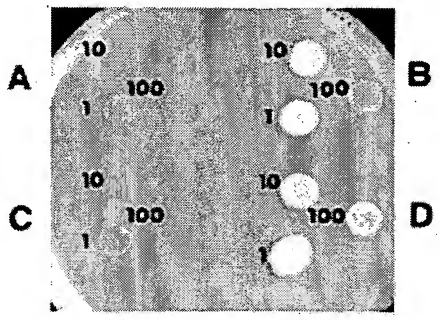

Figure 5. Analysis of the resistance to 5-fluoro-orotate (5-FOA). Resistance to 5-FOA of several yeast strains (lane A, *erg*19::*TRP*1[pPA295-2], lane B, *erg*19::*TRP*1/*ERG*19[pPA295-2], lane C, HC1: *erg*19::*TRP*1[pHC124AT], lane D, *erg*19::*TRP*1/*ERG*19[pHC124AT]) was assayed on minimal medium supplemented with uracil and tryptophan, using three dilutions of yeast cell suspensions (1, 10, 100). The plasmid pPA295-2 bears the yeast functional MVD *ERG*19 gene, and the *URA*3 gene as a selective marker [2]. The haploid and diploid yeast strains bearing the disrupted MVD gene copy with *TRP*1 marker and plasmid pPA295-2 (lanes A and B) were included as negative and positive controls, respectively. Haploid strains which are not able to lose their plasmid do not grow on 5-FOA (lanes A and C). Diploid strains able to lose their plasmid can grow on such a medium (lanes B and D).

haploid disrupted strain transformed with pHC124AT (HC1) could not grow on a 5-fluoro-orotate-containing medium (Figure 5), confirming that this strain was not able to lose its plasmid.

The experiment was repeated, but with pHC126AT, a centromeric (monocopy) plasmid containing the whole MVD coding region under the control of the *PMA1* promoter. The results were identical (data not shown): haploid strains bearing both the yeast deleted copy and the centromeric plasmid with the plant cDNA (HC2) were viable, with a wild-type phenotype, and could not lose their plasmid.

Therefore, we concluded that the cDNA used in this study complements both the yeast mutant defective allele of the *MN19-34* strain and the disrupted MVD gene copy of the TB1Δ strain, confirming that it encodes the *A. thaliana* MVD.

A northern blot analysis was performed on total RNA of yeast complemented with a single copy of the plant cDNA (HC2 strain: *erg19::TRP1*[pHC126AT]). Total RNA (10 and 20 μg) was hybridized with the *A. thaliana* MVD-specific probe presented in Figure 1. The results are shown in Figure 3. For 10 μg of total yeast RNA, a single signal with the expected size was observed, the intensity of which is much higher than the one observed for the plant total RNA. RT-PCR on the complemented yeast total RNA gave a single specific amplified fragment of the expected size (see Figure 3). These results confirmed that the plant cDNA was efficiently expressed in yeast when controlled by the strong *PMA1* promoter.

Sterol analysis

Considering the slow growth rate of the thermosensitive strain complemented with the *At*MVD1 cDNA (HC0: *MN19-34* [pHC124AT]), we decided to measure the amount of sterol in a disrupted back-

Table 2. Quantitative and qualitative analysis of the sterol composition.

Strain	% of 5,7-dienol	Sterol composition (%)			
		Zymosterol	Ergosterol	Fecosterol	Ergosta-5,7-dienol
FL100	0.84 ± 0.06	15	55	5	25
MN19-34	0.15 ± 0.01	0	92	0	8
HC0	0.18 ± 0.03	ND	ND	ND	ND
HC1	0.54 ± 0.03	11	69	3	17
HC2	0.41 ± 0.02	ND	ND	ND	ND

ND, not determined.
The amount of sterol was measured in the haploid strains bearing the disrupted MVD gene copy and complemented with the plant cDNA, HC1 (*erg19::TRP1* [pHC124AT], high-copy-number plasmid) and HC2 (*erg19::TRP1* [pHC126AT], centromeric plasmid), in comparison to the thermosensitive strain *MN19-34* defective for MVD activity, *MN19-34*-complemented strain (HC0: *MN19-34* [pHC124AT]) and the wild-type strain FL100. The strains were grown on complete medium at 28 °C, and the amount of sterol is expressed in mg per 100 mg dry weight. The HC0 strain was grown on minimum medium supplemented with tryptophan. The results are the average of four independent experiments for each strain. The sterol composition of these strains was analysed by gas chromatography (see Materials and methods).

ground. Two independent haploid strains, bearing the deleted MVD allele and complemented with the plant MVD cDNA, carried either on a high copy number plasmid (strain HC1: *erg19::TRP1* [pHC124AT]) or a centromeric plasmid (strain HC2: *erg19::TRP1* [pHC126AT]), were used, and the relative sterol composition was analysed by gas chromatography and compared to the *MN19-34* temperature-sensitive strain, to the HC0 strain and to the wild-type parental strain. For sterol extraction, all these strains were grown at 28 °C in YPD complete medium, except HC0, grown in selective minimal medium (YNB tryptophan) for plasmid maintenance. The results are shown in Table 2. While a typical sterol content of the wild-type strain (FL100) grown in complete medium was about 0.8 mg per 100 mg of dry weight, the amount obtained with HC1 (ca. 0.5–0.6 mg per 100 mg) and HC2 (0.4 mg per 100 mg) did not reach this level. The amount of sterol synthesized increases with the number of copies of the plant cDNA: this amount is significantly higher than the sterol content of the mutant *MN19-34* (ca. 0.15 mg per 100 mg) and the HC0 strain (ca. 0.18 mg per 100 mg) but remains lower than in the wild-type strain. GC analysis showed that the plant cDNA restores a sterol biosynthesis qualitatively comparable to the wild-type strain, since the various intermediates of the pathway can be detected and their respective quantities differ only slightly from the wild-type strain (Table 2).

Two-hybrid assay

In order to evaluate the putative homodimer formation of *A. thaliana* MVD a two-hybrid assay was performed. The *At*MVD1 coding sequence was amplified by PCR and fused in frame to the DNA-binding domain of *lexA* (pLEXA-AtMVD), and to the *GAL4*-activation domain (pGAD-AtMVD). Co-transformants of the L40 recipient strain bearing both plasmids were selected. Transcription of the two reporter genes was then assayed. When the *HIS3* reporter gene is switched on, the strain can grow on synthetic medium lacking histidine (Figure 6a). Transcription of the *lacZ* reporter gene was evaluated both by an XGal assay (Figure 6b) and by measuring β-galactosidase specific activity (Table 3). The results show that the *A. thaliana* MVD forms homodimers *in vivo* (T1 strain), as well as the yeast MVD (Cordier *et al.*, manuscript in preparation). Heterodimer formation between the plant and the wild-type yeast enzymes (T2 strain) was also detected by these assays. By contrast, heterodimer formation could not be detected when the plant enzyme was used in combination with the mutated yeast MVD (Figure 6, Table 3, strains T3 and T4).

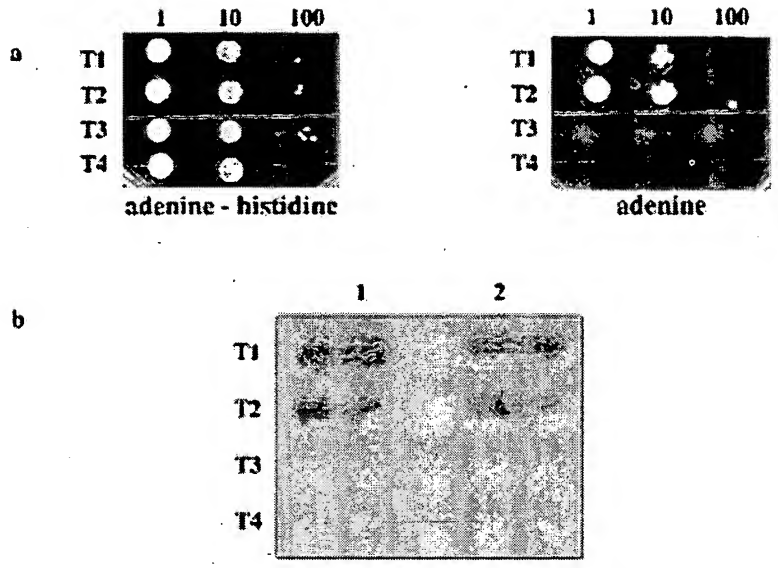

Figure 6. Two-hybrid assay. a. Co-transformed strains T1 to T4 (see Materials and methods) were grown on minimum medium supplemented with adenine and histidine (50 μg/ml) and on minimum medium supplemented with adenine (50 μg/ml), using three dilutions of cell suspensions (1, 10, 100), for the analysis of the activation of the *HIS3* reporter gene. b. XGal assay was performed to analyse the activation of the *lacZ* reporter gene with strains T1 to T4. The blue coloration (dark grey on picture) is indicative of β-galactosidase activity.

Table 3. Measurement of β-galactosidase activity.

Strain	T1	T2	T3	T4	L40
Specific activity	0.314 ± 0.144	0.259 ± 0.104	0.060 ± 0.034	0.058 ± 0.019	0.048 ± 0.012

β-galactosidase activity expressed in nmol of *o*-naphthol per min per mg was measured from protein crude extracts from strains T1 to T4 (see Materials and methods), grown in minimum medium containing 3% (w/v) glucose supplemented with adenine and histidine (50 μg/ml). L40 was included as a negative control. The results are the average of duplicate assays with two independent transformants.

Discussion

With the recent finding of a novel mevalonate-independent pathway in various organisms, it remains to be determined whether these two routes take place in different and totally independent compartments in plants. Isolation and characterization of the cDNAs encoding the enzymes involved in the isoprenoid pathway could allow the study of the heterologous expression of the protein of interest, its purification and the preparation of specific antibodies, in order to elucidate the subcellular localization of the enzymes.

In the mevalonate pathway, IPP is produced by the decarboxylation of MVA-PP. This catalytic step is performed by the mevalonate diphosphate decarboxylase. Based on the study of Toth and Huwyler [25, 26], who identified human, rat and yeast MVDs, we decided to characterise the cDNA encoding the MVD from *A. thaliana*, identified by sequence comparison with databases. Comparison of the sequence of this cDNA with the previously described MVDs revealed that it contains the whole open reading frame encoding this enzyme. The predicted coding sequence displays an overall identity of about 55% with the other eukaryotic MVDs. The best matches were obtained in the N-terminal part of the protein. This cDNA probably encodes a cytoplasmic protein, since no additional transit peptide is predicted at the N-terminus of the protein.

To further characterize the *At*MVD1 gene, the genomic coding region was isolated by PCR from the start to the stop codons. The PCR product is 2837 bp long, and is split into nine exons and eight introns, with 5′ and 3′ splice sites perfectly matching to *A. thaliana* consensus intron splice-sites. A Southern blot analysis suggests the existence of a second

gene encoding MVD in the genome of *A. thaliana*, since both digestions used in this study revealed the presence of additional bands which were not expected according to the restriction map of the *At*MVD1 coding region. These bands could have been due to a partial digestion of the genomic DNA, but this hypothesis was ruled out, since it was not compatible with the restriction maps. Our data suggest a high degree of conservation between the *At*MVD1 gene and its putative homologue since the additional hybridized fragments were observed when high stringency washes were used. The existence of a second MVD gene is consistent with the study of other *A. thaliana* genes involved in isoprenoid biosynthesis. Indeed at least two other enzymes involved in the mevalonate pathway are encoded by two independent genes [6, 13]. In the case of farnesyl diphosphate synthase (FPS), one of the two genes encodes a mitochondrial enzyme [6].

In order to confirm the identity of the protein encoded by this cDNA, its heterologous expression was performed in yeast. To achieve a high level of expression, the *A. thaliana* MVD coding sequence was cloned under the control of a strong yeast promoter, the promoter of the plasma membrane ATPase (*PMA1*) gene, and was introduced into a high-copy-number plasmid. This construct was transformed into a leaky yeast mutant defective for the mevalonate diphosphate decarboxylase (*MN19-34* strain) [4]. This temperature-sensitive strain bears a single-point mutation in the *ERG19* gene encoding the yeast MVD [2]. At the permissive temperature, the ergosterol biosynthetic pathway is functional, but produces very low amounts of 5,7-dienol, i.e. 0.15–0.2% of dry weight, as compared to ca. 0.8–0.9% for a wild-type strain. When introduced into this strain, the *A. thaliana* MVD cDNA clearly suppressed the temperature-sensitive phenotype. However, growth of the complemented strain (HC0) was still significantly impaired in comparison to the wild type, and the amount of sterol produced was not significantly different from the amount of sterol synthesized in the mutant *MN19-34*. In order to further characterize the level of complementation of an MVD-defective yeast and to standardize the growth conditions of the various strains studied, we decided to express the heterologous MVD cDNA in an *ERG19*-disrupted background.

The *erg19::TRP1/ERG19* (TB1Δ) strain was transformed with pHC124AT and haploid progeny harbouring both the null-*ERG19* copy and the *A. thaliana* MVD cDNA was obtained (HC1: *erg19::TRP1* [pHC124AT]), demonstrating that this enzyme is indeed functional in yeast. In addition, the complemented strain could not afford plasmid loss when plated on 5-fluoro-orotate. Moreover, these haploid complemented strains (HC1) grew perfectly well, by contrast to the slow growth of HC0 (*MN19-34* [pHC124AT]).

The sterol content of these strains was studied and compared to both the wild-type and the *MN19-34* temperature-sensitive strain grown in the same medium (YPD). HC1 produces ca. 0.55% of dry weight, whereas *MN19-34* produces only ca. 0.15% and HC0 less than 0.2%. Nevertheless the sterol level does not reach that of the wild-type strain (ca. 0.84%). When we further characterized the sterol composition by GC analysis, we observed that the sterol biosynthetic pathway is functional, since the various intermediates are detected in the complemented disrupted strain (HC1), as well as in the wild type. Indeed, in *MN19-34*, almost only ergosterol is detected showing that the pathway is able to produce the minimal amount of ergosterol necessary for growth. The only noticeable difference in HC1 versus the wild type resides in the ergosta 5,7-dienol/ergosterol ratio: in the wild type, this ratio is higher (about 0.45) than in HC1 (0.24), while it is only about 0.09 in the *MN19-34* strain.

The amount of sterol produced in HC1 is lower than in the wild-type FL100 (Table 1). This could be due to a lower specific activity of the *A. thaliana* MVD. However, it is important to notice that we observed a similar result when the yeast *ERG19* gene was overexpressed [2]. Therefore, it was not excluded that this lower amount of sterol could be the result of a feedback regulation of the sterol biosynthetic pathway when MVD is overproduced. In order to check this hypothesis, the plant MVD cDNA was cloned into a centromeric plasmid, under the control of the same *PMA1* promoter. The *erg19::TRP1/ERG19* (TB1Δ) strain was transformed with this construct, and the haploid complemented strain (HC2) showed the same phenotype as HC1. The amount of sterol in HC2 was lower than in HC1 (0.41% versus 0.54%). By contrast, when TB1Δ was complemented with a single copy of the yeast functional allele, it produces 0.8% of sterol as the wild type [2]. In our case, decreasing the number of copies of the plant cDNA reduces the synthesis of sterols. Therefore, the amount of sterol in yeast strains complemented with the plant MVD cDNA (HC1 and HC2) seems to be the consequence of a lower specific activity of the plant enzyme in yeast

rather than the effect of a feedback regulation due to its overproduction.

The situation observed with the defective *MN19-34* mutant strain is intriguing, since the complementation with the plant cDNA is only partial (HC0), whereas it is almost total for the disrupted strains (HC1 and HC2). The partial complementation phenotype observed in HC0 is well correlated with an apparent lack of increase in the production of sterols. To explain this phenotype, two hypotheses could be proposed. The defective allele of *MN19-34* may titrate the substrate, thereby reducing its accessibility to the plant MVD. Another possibility is that the *erg19-34* allele produces defective subunits that could interact with the plant MVD, thereby altering the enzymatic activity in the HC0 strain. In agreement with the latter hypothesis, MVD of chicken and rat liver [3, 26] and of man (M. Toth, personal communication) have been reported to be homodimeric enzymes. Using a two-hybrid assay, we recently showed that the *Saccharomyces cerevisiae* MVD also forms homodimers whereas the mutated enzyme produced by the *MN19-34* temperature-sensitive strain is severely impaired in dimerization at permissive temperature (Cordier *et al.*, manuscript in preparation). Using the same method, we obtain here evidence for homodimer formation of *A. thaliana* MVD subunits. Moreover, we show that a monomer of the plant enzyme can interact with the wild-type yeast MVD, confirming the high degree of conservation amongst MVD of these species. However we could not detect any interaction between the mutated yeast MVD and the *A. thaliana* monomer. Therefore, the partial complementation in HC0 cannot be explained by the formation of defective plant-yeast heterodimers.

Expression of *At*MVD1 was studied in *A. thaliana* and in the yeast strain HC2 by northern blot analysis and RT-PCR. Expression in plant was shown to be low, since a weak signal was observed, and only for 20 μg of total RNA. Nevertheless, we cannot exclude a tissue-specific expression that would lead to a dilution of the MVD specific mRNA when using total RNA. By contrast, the plant cDNA was efficiently expressed in yeast, since a strong signal was observed with only 10 μg of total HC2 RNA.

The results reported here which describe the molecular characterization of a plant MVD represent a preliminary step to study the compartmentalization and regulation of the mevalonate pathway in higher plants.

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